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benzoxyamino acid azide method according to J. S. Fruton (*8*).

Carbobenzoxy-DL-Serine hydrazide ; m.p., 154°
$C_{11}H_{15}O_4N_3$ (253.2) ; Calculated N 16.6
Found N 16.4

Carbobenzoxy-DL-Serylglycine Benzyl Ester ; m.p., 98°–99°
$C_{20}H_{22}O_6N_2$ (386.4) ; Calculated N 7.3
Found N 7.3

DL-Serylglycine ; m.p. (decomposition), 200°–201°
$C_6H_{10}O_4N_2$ (162.1) ; Calculated N 17.3
Found N 17.1

Oxidation—pH of the reaction mixture was kept neutral during oxidation by the addition of *M*/25 phosphate buffer of pH 8.03 in order to avoid a hydrolysis of the peptide chain. After the oxidation, the excess of periodic acid was removed by filtration as barium salt, and the liberated ammonia contained in the filtrate was determined in the same way as previously described (*9*). The results obtained are shown in Figs. 1–3. As shown in Fig. 1, serine residue in serylglycine was oxidized as rapidly as the free amino acid serine or threonine within only one hour at room temperature. On the other hand, the ammonia liberation from Gly-Ser, Gly-Thr, Gly-Gly, Gly-Ala, Gly-Leu and Gly-Met could be observed only under the condition of heating at 40° or 60°, but practically not at room temperature as shown in Figs. 1–3. Gly-Ser was a little more quickly oxidized than Gly-Thr. Unlike hydroxyamino acid, non-hydroxy-amino acids are little decomposed at room temperature, though oxidized by degrees at a higher temperature as previously reported (*9*). Fig. 3 shows that dipeptides not containing hydroxyamino acid residue were still slower in the reaction.

II. Oxidation Products

As is generally known, by the oxidation of serine and threonine by periodate, NH_3, CHOCOOH and corresponding aldehyde are formed. Analogously a dipeptide containing hydroxyamino acid residue in the form of RCHCHCO-NHCHCOOH such as serylglycine, is expected to produce RCHO, NH_3 and CHOCO-NHCHCOOH on oxidation by periodate. In reality, liberated NH_3 and RCHO were measurable as shown in Fig. 1 and Table II respectively.

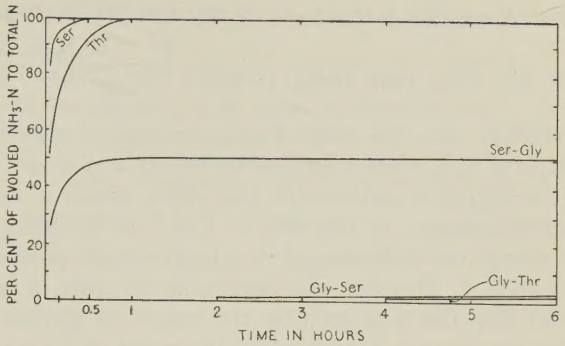

FIG. 1. Oxidation velocities of dipeptides by the action of HIO_4 at room temperature (15°).

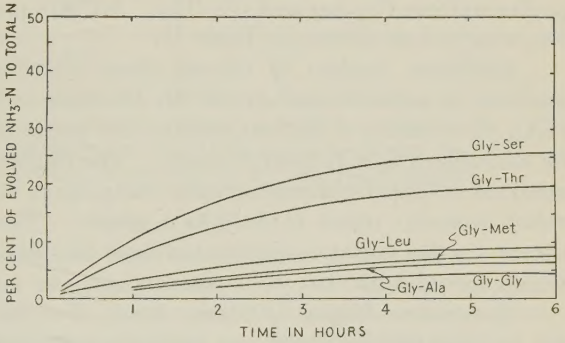

FIG. 2. Oxidation velocities of dipeptides by the action of HIO_4 at 40°.

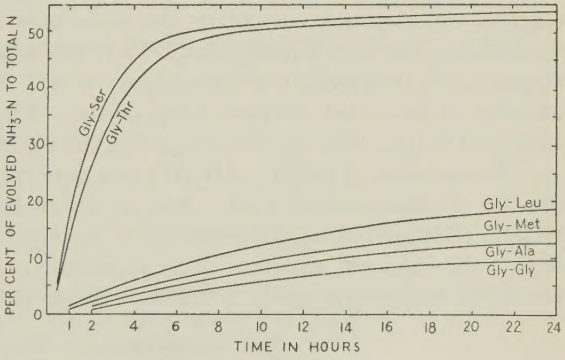

FIG. 3. Oxidation velocities of dipeptides by the action of HIO_4 at 60°.

Figs. 1, 2, 3.—10 mg. of material dissolved in 1 ml. of water and 20 ml. of *M*/25 phosphate buffer of pH 8.03 was oxidized with 1 ml. of 0.5 *M* HIO_4. After the oxidation, excess of HIO_4 was removed as barium salt. The filtrate and washings were combined and NH_3 from them was distilled and determined by using Parnas' apparatus.

A peptide in the form of $NH_2CHCO\text{-}NHCHCHR$, such as Gly-Ser
R′ HOOC OH
or Gly-Thr, may likely produce $NH_2CHCO\text{-}NH_2$, CHOCOOH and
R′
RCHO. On the other hand, synthesized glycine amide liberates one mole of NH_3 when treated with NaOH and steam in Parnas' apparatus. It is therefore conceivable that NH_3, which is determined from the reaction mixture of Gly-Ser, or Gly-Thr by using Parnas' apparatus, is due to the hydrolysis of the glycine amide produced once by the action of HIO_4. Therefore the liberation velocities of NH_3 in case of Gly-Ser, and Gly-Thr will indicate the values of glycine amide formed. This assumption was supported by the result of the analysis of glycine amide produced from Gly-Ser and Gly-Thr. HCHO produced from them was also analysed as shown in Table II.

Qualitative Analysis of Glycine Amide—Gly-Ser and Gly-Thr were oxidized by periodic acid at 60° for 18 hours. After removal of excess HIO_4 by addition of barium acetate, the excess of barium was removed by addition of H_2SO_4 quantitatively. The filtrate was concentrated and analysed by paper-chromatography using upper layer of mixture of acetic acid: butanol: water (1:4:5) as a solvent. The R_f value of the spot shown by the oxidation product of both Gly-Ser and Gly-Thr was 0.19, which agreed with that of synthesized glycine amide.

Quantitative Analysis of Glycine Amidc—For this purpose was utilized the fact that glycine amide was positive to test with biuret reagent. The reaction mixture was treated in the same way and then concentrated to 5 ml. and put into a glass tube. To it was added with 5 ml. of biuret reagent (*10*), warmed in a water bath of 45° for 10 minutes and left standing to be cooled at room temperature. The colour developed was estimated with an electrophotometer and the results are shown in Table I.

Determination of HCHO—HCHO was determined colorimetrically by the use of chromotropic acid. But, as Table II shows, the amount of HCHO estimated from either Gly-Ser or serine at 60° fell short of 45 per cent of the expected amount. This appears to indicate that HCHO once produced underwent partly a further change in the reaction mixture.

DISCUSSION

The assumption that $NH_2CHCO\text{-}NHCHCHR$ might produce
R′ HOOC OH
$NH_2CHCO\text{-}NH_2$, CHOCOOH and RCHO was substantiated by the
R′

result of the determination of HCHO and glycine amide. In the case of

TABLE I

Glycine Amide Produced from Dipeptides by the Action of Neutral Periodate

Material	Amount of glycine amide		
	Calculated	Found	Found / Calculated
	mg.	mg.	%
NH_3 2	0	0	0
DL-Ser 5	0	0	0
Gly-DL-Ser 10	6.83	6.18	91.7
Gly-DL-Thr 10	3.81	3.54	93.2

Each material was oxidized at 60° for 18 hours with 1 ml. of 0.5 *M* HIO_4 in a medium neutralized by addition of *M*/25 phosphate buffer of pH 8.03. After oxidation, excess of HIO_4 was removed and glycine amide in it was determined colorimetrically by using biuret reagent.

TABLE II

Formaldehyde Produced from Serine and Its Peptides by the Action of Neutral Periodate

Material	Conditions of oxidation		Amount of formaldehyde		
	Temperature	Hours	Calculated	Found	Found / Calculated
mg.	°C	hours	mg.	mg.	%
DL-Ser 5	20	3	1.43	1.27	89.1
DL-Ser-Gly 10	20	3	1.66	1.44	87.0
Gly-DL-Ser 8	60	18	1.33	0.23	17.3
DL-Ser 5	60	18	1.43	0.62	43.6

After the oxidation by HIO_4, excess of HIO_4 was removed. Distillate from the residue was diluted properly and HCHO in it was determined colorimetrically by using chromotropic acid.

RCHCHCO-NHCHCOOH, RCHO and NH_3 could be analysed,
HO NH_2 R′
but an attempted determination of CHOCO-NHCHCOOH ended un-
R′

successfully. A paper-chromatography with acetic acid: butanol: water (1:4:5) used as a solvent, showed that no ninhydrin-positive substance was contained in the reaction mixture of Ser-Gly oxidized for 5 hours at room temperature. But it disclosed that the same mixture, hydrolysed by HCl after removal of HIO_4, contained a substance identical in spot with glycine. These facts may be taken to suggest that glyoxyl-glycine, not reacting with ninhydrin, may have been produced from Ser-Gly by the action of HIO_4. Thus the probability that $CHOCO\text{-}NHCH_2COOH$ may be produced from Ser-Gly have been strengthened by those facts.

SUMMARY

1. Dipeptides were oxidized by the action of HIO_4 in a neutral medium.

2. Dipeptide containing hydroxyamino acid residue at its N-terminal was oxidized at 15° as readily as serine itself and gave NH_3, RCHO, and glyoxyl-amino acid as the oxidation products.

3. Dipeptide containing hydroxyamino acid residue at its C-terminal was not easily oxidized, and produced slowly amino acid amide, RCHO and glyoxylic acid when heated.

4. Under such condition of heating, however, oxidation and splitting took place also in dipeptides not containing hydroxyamino acid residue, though they were much slowly oxidized than one containing it.

The authors are indebted to Dr. R. Hirohata, professor of the department, for his valuable advices and encouragement during this work.

REFERENCES

(*1*) Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1939)
(*2*) Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 294 (1941)
(*3*) Rees, M. W., *Biochem. J.*, **40**, 632 (1946)
(*4*) Boyd, M. J., and Logan, M. A., *J. Biol. Chem.*, **146**, 279 (1942)
(*5*) Goebel, W. F., and Perlmann, G. E., *J. Exp. Med.*, **89**, 479 (1949)
(*6*) Desnuelle, P., Antonin, S., and Casal, A., *Bull. Soc. Chim. Biol.*, **29**, 694 (1947)
(*7*) Jansen, E. F., Curl, A. L., and Balls, A. K., *J. Biol. Chem.*, **189**, 671 (1951)
(*8*) Fruton, J. S., *J. Biol. Chem.*, **146**, 463 (1942)
(*9*) Arakawa, K., *J. Biochem.*, **44**, 217 (1957)
(*10*) Weichselbaum, T. E., *Am. J. Clin. Path.*, **10**, 40 (1946)

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METABOLISM OF PARA-AMINOSALICYLIC ACID

III. A COMPARATIVE STUDY AMONG DIFFERENT KINDS OF MAMMALS

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(Received for publication, March 6, 1957)

In the previous papers (*1*, *2*), nine metabolic products of *p*-aminosalicylic acid (PAS) which were excreted in human urine were described. In the present paper, experimental results are presented to reveal a remarkable difference, both qualitative and quantitative, in metabolic pattern of PAS among different kinds of mammals such as human being, rabbit, dog, rat and mouse respectively.

EXPERIMENTAL AND RESULTS

Human beings and dogs were fed with daily mixed diet, rabbits with vegetables and grains, and mice and rats with pressed barley. One tenth gram of PAS per kilogram body weight was administered to human beings, dogs and rabbits either orally or by intravenous injection, but no appreciable difference was recognized with respect to administration methods in each species. The same dose of the drug was administered to rats and mice by intraperitoneal injection. The urine excreted over 6 hours was collected, concentrated under reduced pressure to less than one tenth of the original volume, and desalted by adding ethyl alcohol. Definite portions of the samples were submitted to paper chromatography in butanol-acetic acid-water (4:1:5) and methanol-benzene-butanol-water (2:1:1:1) and the chromatograms were sprayed with Bratton-Marshall's reagents, ferric chloride solution or Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) in order to separate and to characterize the substances as described in the first paper (*1*).

Identification of each spot obtained from the urine of one species with the similar spot obtained from the urines of other species was achieved by determining the R_f values in the two solvents, the ultraviolet absorption curves and the colors developed after spraying with the

reagents.

The urine samples obtained from 13 men, 10 of which were the patients with slight pulmonary tuberculosis, 6 samples each from dogs, rabbits and mice, and 2 from rats were tested. Any qualitative difference could not be found among the samples from the same species.

Only human urine gave all the nine spots corresponding to unchanged PAS, N-acetyl-PAS, *p*-aminosalicyluric acid, a sulfate conjugate, OH-glucuronides of PAS and N-acetyl-PAS, COOH-glucuronides of PAS and N-acetyl-PAS and a glucuronide of an unknown derivative of PAS as reported in the previous papers (*1*, *2*). Among these nine com-

TABLE I

Metabolic Products of p-Aminosalicylic Acid in the Urine of Different Kinds of Mammals

Metabolic products of PAS	R_f*	Man	Rabbit	Mouse	Rat	Dog
N-Acetyl-PAS	0.83	###	###	###	###	−
PAS	0.79	###	###	###	###	###
p-Aminosalicyluric acid	0.46	##	−	−	−	−
COOH-Glucuronide of N-acetyl-PAS	0.38	##	±	±	±	−
OH-Glucuronide of N-acetyl-PAS	0.33	+	±	±	±	−
COOH-Glucuronide of PAS	0.28	###	###	###	###	###
Sulfate conjugate	0.28	+	+	−	−	##
Unknown glucuronide	0.19	+	+	##	##	+
OH-Glucuronide of PAS	0.14	+	##	±	±	±

* Chromatogram run in butanol-acetic acid-water (4 : 1 : 5).

pounds, unchanged PAS, N-acetyl-PAS and COOH-glucuronides of PAS and of N-acetyl-PAS overwhelmed other substances in the size and the intensity of their spots, as shown in Table I. In some experiments, the urines were collected every 15 minutes after the administration of PAS, the volumes were adjusted as to correspond to 2/3 ml. per minute excretion, and a definite amount of each sample was submitted to a paper chromatography. The chromatograms obtained showed some quantitative difference from each other, the urine samples collected from 5 to 7 hours after administration of the drug giving comparatively large spots of sulfate conjugate and N-acetyl-PAS.

The dog urine lacked glycine conjugate, all of the N-acetyl compounds and OH-glucuronide of PAS, while the COOH-glucuronide was abundant and the sulfate conjugate was excreted in larger quantity than in the other species. No trace of *p*-aminosalicyluric acid could be detected even when the dog was fed with high protein diet.

In rabbit urine *p*-aminosalicyluric acid and COOH-glucuronide of N-acetyl-PAS were not found. In rat urine and mouse urine none of *p*-aminosalicyluric acid, both types of glucuronide of N-acetyl-PAS and the sulfate conjugate were detected (Table I).

DISCUSSION

As shown in Table I, COOH-glucuronide was formed in considerable large quantities in all of the species examined. Tsukamoto and Yamamoto (*3*) reported that the urine of rabbits given PAS contained a glucuronide of PAS, though they did not clarify the position of conjugation. The glucuronide they found is most probably COOH-glucuronide of PAS judging from its R_f and hydralyzability with alkali. On the other hand, OH-glucuronide was found in less amount in the urine of human beings and rabbits, and was not or only in small amount in the urines of dogs, rats and mice. This fact suggests that there is some difference in the mechanism of glucuronide formation of both types.

As it has been recognized that aromatic amines are not acetylated in dog, none of the N-acetyl compounds derived from PAS was found in dog urine. But sulfate conjugate was excreted in the urine of dogs administered PAS in a relatively large amount compared with other species. It is noteworthy that glycine conjugate of PAS could not be detected in dog urine, while that of *p*-aminobenzoic acid was undoubtedly demonstrated in the urine of dog after dosing *p*-aminobenzoic acid as will be reported later. Moreover, an appreciable amount of *p*-aminosalicyluric acid was excreted only in human urine. Such marked differences observed in metabolic pattern of PAS may be attributed to the difference of food. But it seems not the case because the dog fed with high protein diet did not form the glycine conjugate.

Kawamata and Hiratani (*4*) reported that rat excreted a glutamine conjugate after administration of PAS as human beings did (*5*). Any attempt to detect the glutamine conjugate was not successful in all the species examined.

SUMMARY

A distinct qualtative difference in the metabolic pattern of PAS was demonstrated among human being, dog, rabbit, rat and mouse.

Man excreted all the nine metabolic products. Dog excreted PAS, COOH-glucuronide, sulfate conjugate and the unknown glucuronide; rabbit PAS, N-acetyl-PAS, COOH-glucuronide, OH-glucuronide and the unknown glucuronide; rat and mouse PAS, N-acetyl-PAS, COOH-glucuronide and the unknown glucuronide.

REFERENCES

(*1*) Nakao, M., *J. Biochem.* (*Japan*), **44**, 327, (1957)
(*2*) Nakao, M., and Yanagisawa, I., *J. Biochem.* (*Japan*), **44**, 433 (1957)
(*3*) Tsukamoto, H., and Yamamoto, A., *Pharm. Bull.* (*Japan*), **3**, 427 (1955)
(*4*) Kawamata, J., and Hiratani, T., *Med. J. Osaka Univ.*, **6**, 417 (1955)
(*5*) Kawamata, J., and Kashiwagi, K., *Med. J. Osaka Univ.*, **6**, 111 (1955)

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ON THE KINETICS OF THE AMIDASE ACTIVITY OF TRYPSIN

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(Received for publication, April 2, 1957)

Trypsin is known to catalyze not only the hydrolysis of denatured protein, but also the hydrolysis of amide or simple alkyl ester of benzoyl-L-arginine (*1–4*), and in addition it can catalyze the transpeptidation reaction (*5*). It is a very interesting problem that a single enzyme of trypsin catalyzes several different reactions, the problem is concerned the nature of its active center and the reaction mechanism of its catalysis.

Recently, the property of the active surface of trypsin was discussed by Gutfreund (*6*), who assumed an imidazole group of histidine as a functional group located on its active surface, and that free from the combination of both the enzyme and substrate at a " catalytic site ", and there is a strong binding power on the " specificity site " of the side chain of arginine.

In order to analyze this property of trypsin, we made an experiment to study kinetically the trypsin catalyzed reaction. In this paper, the course of the reaction and the effects of *pH* and temperature toward the reaction will be described from the kinetical point of view.

EXPERIMENTAL

Enzyme—Crystalline trypsin was prepared from beef pancreas according to the method of Kunitz and Northrop (*7*), and stored in cold room. Stock solution was prepared by dissolving crystalline trypsin to 100 mg. of protein 100 ml. in $N/100$ H_2SO_4 and was used within three days during which it was stable. Just before the addition to substrate solution, this stock solution was adjusted to required *pH* and then diluted up to the volume of 50 mg. of protein 100 ml. 0.2 ml. of this solution was used for the measurement of enzyme activity.

Substrate—α-Benzoyl-L-arginine amide hydrochloride (BAA) was prepared from L-arginine hydrochloride according to the method of Bergmann *et al.* (*2*), and Schwert *et al.* (*1*). The nitrogen content of this material was 20.03 per cent (theoretical value for BAA·HCl·H_2O, 331.8 ; N, 21.09 per cent).

Measurement of Enzyme Activity—The amidase activity of trypsin was measured by the Conway micro diffusion technique. The detailed procedure was performed according

to the method described by Schwert *et al.* (*1*). *M*/10 Phosphate buffer was used.

RESULTS

I. The Course of the Reaction

In order to test the course of trypsin catalyzed hydrolysis of BAA, the relationship between the activity of trypsin on various concentrations of BAA and the time was investigated. The activity measurements

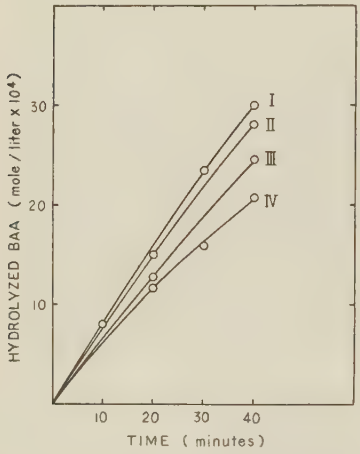

Fig. 1. The relationship between trypsin activity and time. *pH* =7.5 ; temperature=30° ; [trypsin] =83 μg. prot./ml. The numbers denote the following : I, 0.0416 *M* BAA ; II, 0.0208 *M* BAA ; III, 0.0104 *M* BAA ; IV, 0.0067 *M* BAA.

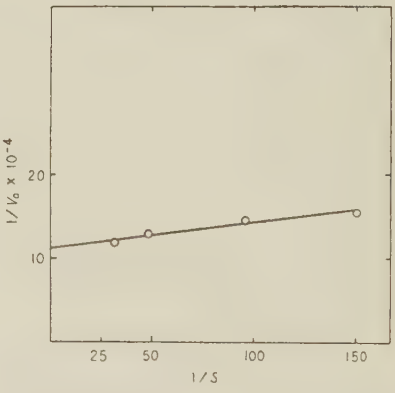

Fig. 2. Lineweaver-Burk plot.

were made at four levels of BAA concentration. As may be seen in Fig. 1, the course of the reaction was found to follow linear relation within 30–40 minutes. Lineweaver-Burk relation was obtained linear as shown in Fig. 2, from which the Michaelis constant was calculated as $Km=3.18\times10^{-3}$ mole/litre. This value is close to that of Bernhard (*8*) and greater than that of Niemann (*9*).

The rate constants of the reaction at each substrate concentration were calculated from application of conventional first order equation.

These values were shown in Table I. The k values increased with decreasing concentration of substrate. This fact reveals that the trypsin catalyzed hydrolysis of BAA follows to more complex than the simple first

TABLE I

k Calculated from Application of Simple First Order Kinetics

BAA (*mole, liter*$^{-1}$)	k (*mole, liter,*$^{-1}$ *min.*$^{-1}$)
0.0416	1.9×10^{-3}
0.0208	$3.7\times$,,
0.0104	$6.6\times$,,
0.0067	$9.3\times$,,

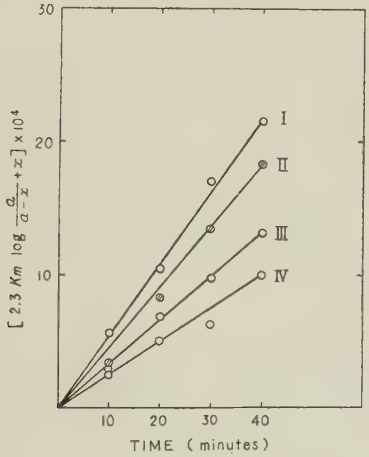

FIG. 3. Plot of $[2.3\cdot Km\cdot\log\frac{a}{a-x}+x]$ to the time. [BAA]=0.05 *M* ; *pH*=7.5 ; temp.=30°. The numbers in figure denote following trypsin concentration:

I, 83μg. prot./ml. ; II, 66.4 μg. prot./ml. III, 49.8 μg. prot./ml. ; IV, 33.2 μg. prot./ml.

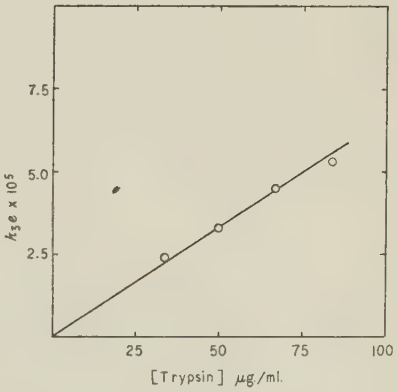

FIG. 4. Plot of k_3e to trypsin concentration. Temp.=30° ; *pH*=7.5 ; [BAA]=0.05 *M*.

order formulation. Elkins-Kaufman and Neurath (*10*) have proposed the following integrated form of Michaelis-Menten equation to account for the order of enzyme-catalyzed reactions.

$$k_3e=\frac{1}{t}[2.3\cdot Km\cdot\log\frac{a}{a-x}+x] \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

In this equation, e is the total concentration of enzyme, a the initial concentration of substrate, x the splitted substrate concentration at any time, t, and k_3 the rate constant of splitting reaction of enzyme-substrate

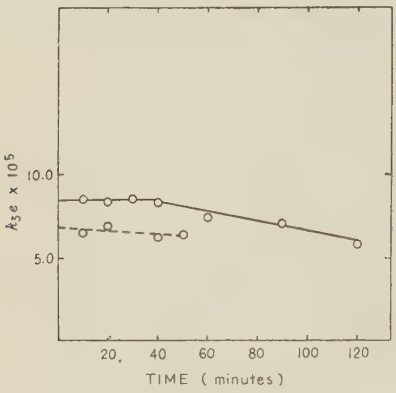

Fig. 5. Plot of k_3e to the time. Temp.=30° ; pH=7.5.

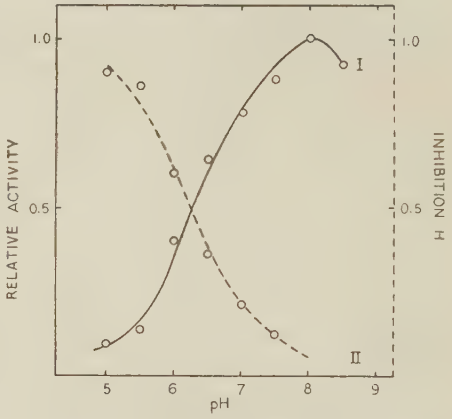

Fig. 6. The pH dependence on the activity of trypsin. [BAA]=0.03M ; temp. =30° ; pH=7.5.

Table II

k_3e Calculated from Application of Eq. (1) at Various Substrate Concentrations

BAA (*mole, liter*$^{-1}$)	k_3e (*min.*$^{-1}$)
0.0417	8.4×10^{-5}
0.0208	$8.7\times$,,
0.0104	$8.6\times$,,
0.0067	$8.3\times$,,

complex.

The values of k_3e shown in Table II were calculated from application of equation (1). These values are constant at all of the substrate concentrations. The relationships between $[2.3\cdot Km\cdot\log\ a/(a-x)+x]$ and the time or k_3e and e are also linear as shown in Figs. 3 and 4. But

when k_3e was calculated from the rate at above 50 minutes or the rate measured at the low substrate concentration, it decreased as shown in Fig. 5. Assuming a molecular weight of 17,000 for trypsin, k_3 was calculated as 11 minute^{-1}.

II. The pH Dependence of the Activity of Trypsin

The relation of *pH* against the trypsin catalyzed hydrolysis of BAA is recorded in Fig. 6, Curve I. *pH* Optimum of the reaction is 8.0. The broken line (curve II) in Fig. 6 is the relation between the inhibition degree of trypsin catalyzed hydrolysis by hydrogen ion and the concentration of hydrogen ion. This curve is in agreement with the theoretical dissociation curve of $pK=6.28$. And Michaelis constant is not affected by hydrogen ion as seen in Table III.

III. The Effect of Temperature on the Reaction

It would be interesting to observe the temperature dependence of

TABLE III

The Values of Km at Various pH

pH	*Km* (*mole, liter*$^{-1}$)
8.0	3.3×10^{-3}
7.5	$3.18\times$ „
6.5	$3.3\times$ „

Km, K_H' and k_3. Data plotted in Figs. 7, 8 and 9 show the change of these values with temperature at *pH* 7.5. ΔH, $\Delta H'$ and ΔH^* calculated from these date are 5.9, 7.1, and 12.3 Kcal. mole^{-1}, respectively. ΔH^* was calculated from the equation $\Delta H^*=\Delta E-RT$.

DISCUSSION

It will be assumed from the linear relation of Lineweaver-Burk plot recorded in Fig. 2 that the trypsin catalyzed hydrolysis of BAA follows to the simple Michaelis-Menten equation,

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$ES \xrightarrow{k_3} E+P \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

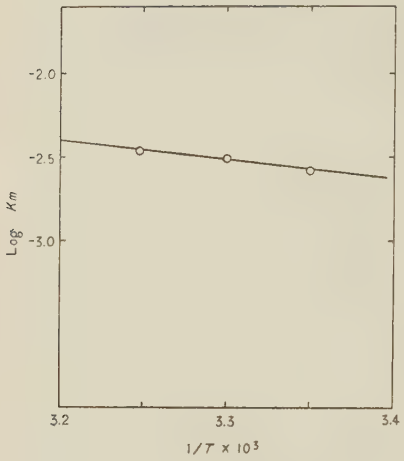

FIG. 7. Plot of log Km to $1/T$ at pH 7.5.

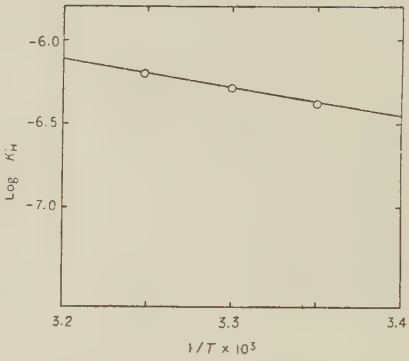

FIG. 8. Plot of log K_H' against $1/T$ at pH 7.5.

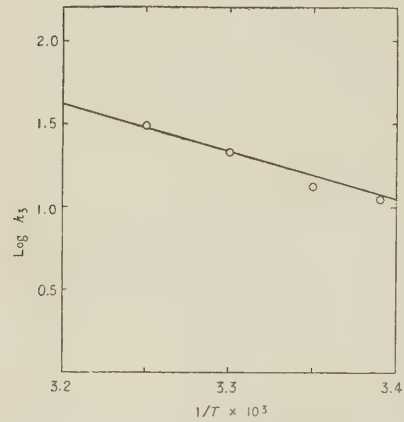

FIG. 9. Plot of log k_3 against $1/T$ at pH 7.5.

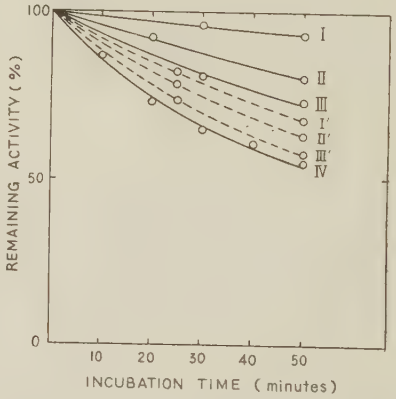

Fig. 10. The relationship between the remaining activity of trypsin and incubation time at pH 7.5, 30°:

The numbers in figure denote following: I, 5×10^{-3} M BA; II, 2×10^{-3} M BA; III, 1×10^{-3} M BA; I′, 5×10^{-3} arginine; II′, 2.5×10^{-3} M arginine; III″, 1×10^{-3} M arginine; IV, control.

where E, S and P are trypsin, BAA and BA, respectively. In this equation, it is interested that the velocity constant which was calculated from the integrated formulation of Michaelis-Menten equation is obtained at constant. Elkins-Kaufman and Neurath (*10*) have observed the same phenomenon on carboxypeptidase catalyzed reaction. Therefore, it may be expected that both reaction of trypsin and carboxypeptidase is kinetically analogeous.

Since trypsin is rapidly inactivated in a definite *pH* region, and inhibited by benzoyl-L-arginine (BA), it has to be checked on whether the rate of hydrolysis observed in this experiments is affected by such reaction. But these effects are not plausible, because k_3 was obtained as constant under this experimental condition. This assumption that the rate of the hydrolysis of BAA will not be affected by self-inactivation of trypsin nor inhibited by BA may be supported also by the fact shown in Fig. 10. That is, if trypsin is incubated with BA in room temperature before it is added to substrate solution, the self-inactivation of trypsin is remarkably protected by BA. And this protection decreases with decreasing of the concentration of BA. Therefore, it is expected that BAA may also protect trypsin from its self-inactivation, in other words, this self-inactivation does not take place in the existence of BAA. It is interested to note that the protective action by arginine chloride is not so remarkable.

From the results shown in Fig. 6 and Table III, it is assumed that the change of trypsin activity with the change of hydrogen ion concentration is to be ascribed to the ionization of a ionizable group of active surface of the enzyme. Since the relation between the inhibition of trypsin by hydrogen ion and its concentration is expressed as a sigmoid curve of first order, this inhibition reaction may be caused by the association of a proton to the ionizable group of trypsin. And it may be of a non-competitive nature, because Michaelis constant is not affected by the change of hydrogen ion concentration. The apparent ionization constant $pK'_H=6.28$ and the heat of ionization of this group are very close to the figures quoted by Gutfreund (*6*) who obtained it from the kinetical studies of the trypsin catalyzed hydrolysis of benzoyl-L-arginine ethyl ester. He assumed from the remarkable agreement of his data with the ionization constant and the heat of ionization of imidazol of histidine, that the functional group of trypsin will be imidazol group of histidine. Although the active center of trypsin would not be decided only from these values, yet it is plausible that the active surface of trypsin catalyzing both BAA and benzoyl-L-arginine ethyl ester, might be on

the same site, because our data are in good agreement with the data quoted by Gutfreund (*6*).

The heat of activation (ΔH^*) can be obtained from the temperature dependence of the rate constant k_3 in the rate determining step.

Now, if *Km* is the dissociation constant of ES intermediate complex, the equilibium constant *K* of the ES forming reaction is expressed by the reciprocal of *Km*. Then, the heat of reaction of ES formation can be obtained from the result shown in Fig. 7. Thus, the thermodynamical constants of trypsin catalyzed reaction are summarized in Table IV.

It is interested in these values that the entropy change in the ES forming reaction is positive and negative in the activated state. These entropy changes in this reaction are very similar to the figures quoted by Laidler (*11, 12*) in the pepsin and urease catalyzed reactions. This phenomenon might be suggesting the change in the configuration of enzyme molecule during the course of the trypsin catalyzed reaction.

TABLE IV

The Thermodynamical Constants of the Trypsin Catalyzed Hydrolysis of Benzoyl-L-arginine Amide

ES formation	ES activation
$\Delta H = 5.9$ Kcal./mole	$\Delta H^* = 12.3$ Kcal.
$\Delta F = -3.5$,,	$\Delta F^* = 16.6$,,
$\Delta S = +31.0$ cal./mole/deg.	$\Delta S^* = -14.1$ cal./mole/deg.

SUMMARY

1. It has been shown that the hydrolysis of benzoyl-L-arginine amide by trypsin does not follow the conventional first order kinetics. The constant value of k_3 which is independent of both enzyme and substrate concentration was obtained from integration of Michaelis-Menten equation.

2. The existence of a group with $pK'_H = 6.28$ at 30° and the heat of ionization = 7.1 Kcal. mole^{-1} in trypsin molecule was assumed from the *pH* dependence on the catalytic activity of trypsin. These values were very close to the figures quoted by Gutfreund.

3. The temperature dependence of the trypsin catalyzed reaction was observed and the heats of enzyme-substrate complex formation and

that of the activation were calculated as 5.9 and 12.3 Kcal. mole^{-1}, respectively.

The authors indebted to Prof. K. Kaziro for his continuous help and stimulating discussions. The authors also express their gratitude to the Ministry of Education for the aid of the Scientific Research Fund.

REFERENCES

(*1*) Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172**, 221 (1948)
(*2*) Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939)
(*3*) Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130**, 81 (1939)
(*4*) Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **138**, 243 (1941)
(*5*) Waley, S. G., and Watson, J., *Biochem. J.*, **57**, 529 (1954)
(*6*) Gutfreund, H., *Trans. Farad. Soc.* : **51**, 441 (1955)
(*7*) Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936)
(*8*) Bernhard, S. A., *Biochem. J.*, **59**, 506 (1955)
(*9*) Harmon, K. M., and Niemann, C., *J. Biol. Chem.*, **178**, 743 (1949)
(*10*) Elkins-Kaufman, E., and Neurath, H., *J. Biol. Chem.*, **175**, 893 (1948)
(*11*) Laidler, K. J., *J. Amer. Chem. Soc.*, **72**, 2489 (1950)
(*12*) Casey, E. J., and Laidler, K. J., *J. Amer. Chem. Soc.*, **72**, 2159 (1950)

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STUDIES ON XANTHURENIC ACID

XV. EFFECT OF XANTHURENIC ACID ON HEXOKINASE ACTIVITY

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(Received for publication, April 2, 1957)

As previously reported (*1*), xanthurenic acid acts as one of diabetogenic substances. The mechanism of its action has been studied from various angles. By making use of albino rats administered with it, Kotake and Nagayama (*2*) detected a remarkable increase of the inorganic phosphate in blood and liver, but a marked decrease of the creatine-phosphate content in liver.

They further discovered that any temporary decrease of blood inorganic phosphate usually appeared in case of the glucose injection could not be observed when xanthurenic acid was simultaneously administered.

The authors considered it highly possible from these findings that hexose phosphorylation, at the first step of carbohydrate metabolism, might be inhibited by xanthurenic acid.

In pursuit of this line of research, the quantitative determination of fructose-6-phosphate and fructose-1,6-diphosphate in liver of the rat treated with xanthurenic acid were carried out, and compared with those of normal animals.

It was found that the remarkable decrease of these two substances occurred in the xanthurenic acid injected rats. Further the authors detected the effect of xanthurenic acid upon yeast or animal hexokinase and found the enzyme activities were greatly inhibited by this acid. It is to be noted in this connection that the experiment of the inhibition of animal hexokinase activity by alloxan was already done by M. Grifth in 1949. (*3*)

EXPERIMENTAL AND RESULTS

I. Quantitative Determination of Fructose-6-phosphate and Fructose-1-6-diphosphate

Albino rats each weighing about 200 g, were fed on a synthetic diet

of the composition as follows ; casein 22 per cent, agar-agar 3 per cent, starch 52 per cent, sugar 5 per cent, Mc Collum salt mixture 6 per cent, buttur 10 per cent, yeast 2 per cent.

Each one of these animals was injected once or twice a day with 300 mg. (per kg. of body weight) of xanthurenic acid, and killed by decapitation after 24 hours, and the liver was cut out.

On this fresh material, the contents of fructose-6-phosphate and fructose-1-6-diphosphate were determined by the Roe's method (*4*) and compared with those of normal rats.

TABLE I

Effect of Xanthurenic Acid on Phosphorylation

No.	Control	Xanthurenic acid injected (300 *mg./kg.*)		Tryptophan (0.1 *g.*) sodium butyrate (0.4 *g.*) administered
		Once	Twice	
The amount of fructose-1-6-diphosphate *mg.*/100 g.				
1	24.5	16.0	20.1	21.3
2	27.0	18.3	18.6	18.0
3	25.0	17.0		18.5
Average	25.5	17.1	19.3	19.2
The amount of fructose-6-phosphate				
1	12.9	9.0	9.2	10.2
2	13.24	7.66	8.06	9.34
3	11.52	7.38	7.8	10.00
Average	12.55	8.01	8.35	9.84

In the other group, tryptophan and sodium butyrate were administered instead of the xanthurenic acid.

It is clear from Table I that the hexose phosphorylation was inhibited to appreciable extent by the administration of xanthurenic acid or by giving tryptophan and sodium butyrate.

II. Experiments on Hexokinase Activity

(*a*) *Preparation of Yeast Hexokinase*—The preparation of yeast hexokinase was conducted in accordance with the Okunuki's (*5*) method.

An aliquot amount of dried bread yeast was suspended in three

times as much as 1/20 *M* phosphate buffer (pH 7.0) and shaken for three hours at 35° under constant precaution to keep the acidity of the solution to pH 7.0 by adding KOH. Then it was cooled at 0° and subjected to filtration. The filtrate was mixed with 350 g. of ammonium sulfate per 1 liter and the resulting precipitates were removed by means of filtration.

To the supernatant fluid, 138 g. of ammonium sulfate per 1 liter was added again (0.55–0.75 saturation) and the precipitate was corrected by centrifuge. The precipitate was dissolved in water and subjected again to the same procedure as described above.

(*b*) *Preparation of Animal Hexokinase*—The preparation of animal hexokinase was carried out almost in accordance with the Stein and Cori's (*6*) method. By means of cooled mixer, the pieces of dog's muscle that was prepared by grinding well suspended in 1.5 times as much as water and kept aside for five minutes.

Next, the extract of this mixture was quickly filtered through the gauze and the filtrate was subjected to centrifugal precipitation and 30 per cent acetone fraction of the supernatant regulated pH 6.2 (by 1/10 *N* HCl) was employed for the experiment.

(*c*) *Effect of Xanthurenic Acid on the Activities of Animal and Yeast Hexokinase*—An aliquot amount of animal and yeast hexokinase preparations were dissolved in 2 ml. of the reaction mixtures of the composition as follows;

	Animal hexokinase			*Yeast hexokinase*	
main :	hexokinase	in final	main :	hexokinase	in final
	$MgCl_2$	0.008 *M*		$MgCl_2$	0.008 *M*
	CH_2ICOOK	0.002 *M*		$KHCO_3$	0.027 *M*
	KF	0.053 *M*		Glucose	0.01 *M*
	Glucose	0.01 *M*			
side :	ATP	0.004 *M*	side :	ATP	0.008 *M*

(Total volume is 2.0 ml.)

Hexokinase was dissolved in the solution of glucose as the substrate in such a concentration that there would be no effect of the presence of myokinase, (adenylate kinase). In accordance with the activity of the enzyme, 0.004 *M* ATP was added to animal hexokinase and 0.008 *M* to yeast hexokinase respectively.

On this occasion, xanthurenic acid having each different concentrations was employed in dissolving it either in $KHCO_3$ or $NaHCO_3$. The vessel was filled with N_2-gas and shaken in the thermostat strictly kept

at 37°. The liberated amount of carbon dioxide were read with the aid of Warburg's manometer. (CO_2 : N_2=5 :95) The results are illustrated in Figs. 1 and 2.

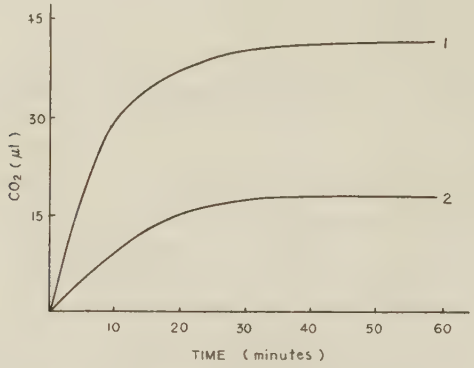

Fig. 1. Animal hexokinase.
1 : control. 2: in final xanthurenic acid 10^{-3} *M*.

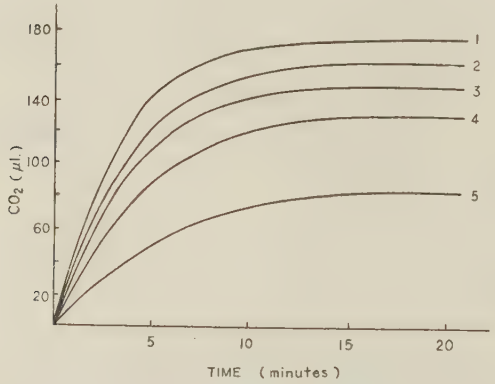

Fig. 2. Yeast hexokinase. 1 : in final xanthurenic acid 10^{-6} *M*, 2 : control, 3 : in final xanthurenic acid 10^{-5} *M*, 4 : in final xanthurenic acid 10^{-4} *M*, 5 : in final xanthurenic acid 10^{-3} *M*.

It will be noted that both hexokinases were inhibited in the presence of xanthurenic acid except the case where its end concentration was 10^{-6} *M*.

It is to be noted, however, that contrary to the expectation, xanthurenic acid in its final 10^{-6} *M* concentration resulted in activating the said hexokinase activity.

(*d*) *Effect of 4-Hydroxy-8-methoxy-quinolin-2-carboxylic-acid* (*7*), *Kynurenic Acid, Anthranilic Acid and 5-OH-Anthranilic Acid* (*8*)—These substances have been proved by Kotake and his coworkers to suppress the diabetogenic of xanthurenic acid. It was tested here whether such antagonistical relation keeps with the effect upon yeast hexokinase activity. This experimental procedure is quite the same as one in the foregoing experiment, except that the reactions mixture contained an same amount of the test substance as that of xanthurenic acid. The results are il-

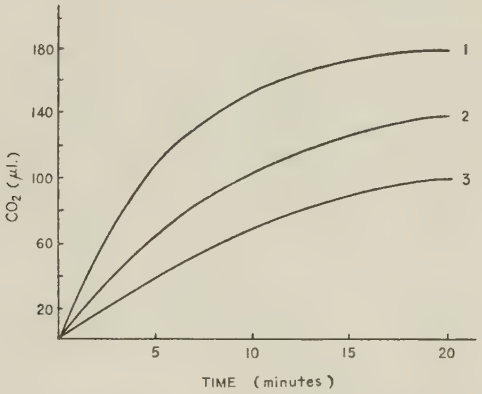

Fig. 3. 1 : control, 2 : xanthurenic acid 10^{-8} *M*+4-OH-8-OCH_3-quinolin-2-carboxylic acid 10^{-8} *M*, 3 : xanthurenic acid 10^{-8} *M*.

lustrated in Figs. 3, 4 and 5.

It is clear from these figures that the substances which suppressed the diabetogenic of xanthurenic acid also were active in protecting hexokinase activity from the inhibition by xanthurenic acid.

(*e*) *Effect of Cysteine on Hexokinase Activity*—In an attempt to clarify the mechanism of the inhibition against hexokinase activity, another and final experiment was carried out.

Since EDTA is a strong chelating agent, however, it is considered that its some more higher concentration may inhibit hexokinase activity. The authors' experiment, as shown in Fig. 6, proved that 10^{-3} *M* EDTA inhibited remarkably hexokinase. Further it was confirmed that the

simultaneous addition of an equal mole of cysteine could protect the enzyme against such inhibition.

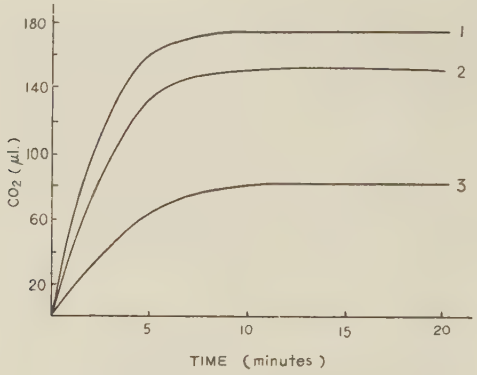

FIG. 4. 1 : control, 2 : xanthurenic acid 10^{-3} *M*+kynurenic acid 10^{-3} *M*, 3 : xanthurenic acid 10^{-3} *M*.

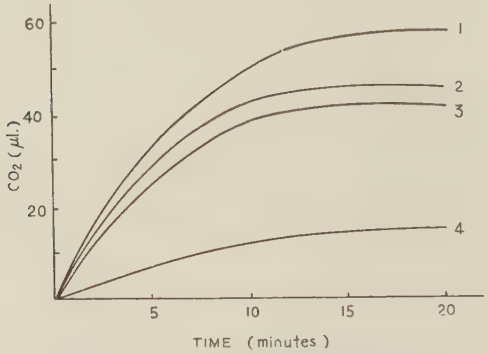

FIG. 5.

1 : xanthurenic acid 10^{-3} *M*, anthranilic acid 10^{-3} *M*)+
2 : xanthurenic acid 10^{-3} *M*, 5-OH-anthranilic 10^{-3} *M*)+
3 : control
4 : xanthurenic acid 10^{-3} *M*

Enzyme system.

Main :	hexokinase	
	$MgCl_2$	0.008 *M*
	$KHCO_3$	0.027 *M*
	Glucose	0.02 *M*
Side :	ATP	0.008 *M*
		in final

total volume 2.0 ml.

Xanthurenic acid can also be considered to be a chelating agent, then xanthurenic acid is experimented in the same way. Hence it was

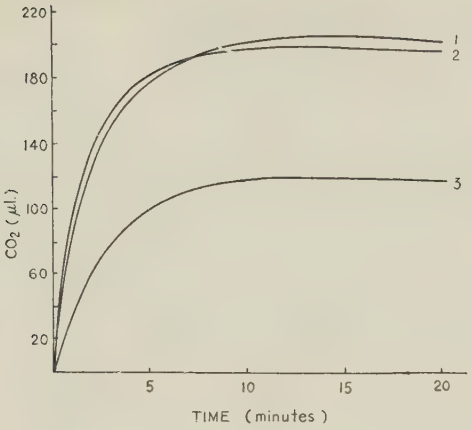

FIG. 6. 1 : control, 2 : EDTA 10^{-3} *M*+cysteine 10^{-3} *M*
3 : EDTA 10^{-3} *M*.

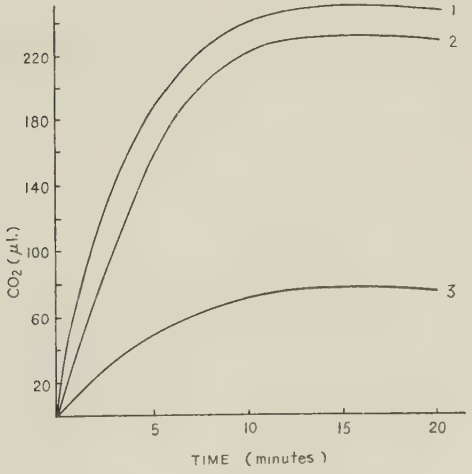

FIG. 7.

1 : control

2 : cysteine 10^{-3} *M*, xanthurenic acid 10^{-3} *M*) +

3 : xanthurenic acid 10^{-3} *M*

Enzyme system

Main : hexokinase

$MgCl_2$	0.008 *M*
$KHCO_3$	0.027 *M*
Glucose	0.02 *M*

Side : ATP 0.009 *M* in final total volume 2.0 ml.

tested here whether cysteine is also effective in checking inhibitive action of xanthurenic acid on hexokinase (Fig. 7).

SUMMARY

From the experiments stated above, xanthurenic acid can unmistakably inhibit hexokinase activity and it is highly possible to imagine that what has much to do with the inhibition may be either Mg^{++} or —SH system. In either case, however, it is safe to consider that since glycosuria due to xanthurenic acid can be found to be identical with glycosuria caused by the action of alloxan, xanthurenic acid has been confirmed to be a diabetogenic substance.

1. Xanthurenic acid inhibits phosphorylation of hexose in rat liver.
2. It inhibits also animal and yeast hexokinase activity.
3. 4-OH-8-OCH_3-quinolin-2-carboxylic acid, kynurenic acid, anthranilic acid, 5-OH-anthranilic acid and cystein protect hexokinase activity against the inhibitive action of xanthurenic acid.

The authors wish to express their sincere thanks to Prof. Y. Kotake for his constant encouragement and to Prof. K. Kodama for his kind revision and to Prof. K. Okunuki, Mr. K. Iwasa and Mr. F. Imamoto of the Department of Science of the Osaka University for their kind assistance in the preparation of hexokinase.

REFERENCES

(*1*) Kotake, Y. (Jr.), and Inada, T., *Proc. Japan Acad.*, **28**, 65 (1952)
(*2*) Kotake, Y. (Jr.), and Nagayama, I., *Proc. Japan Acad.*, **31**, 311 (1955)
(*3*) Grifth, M., *Arch, Biochem.*, **20**, 451 (1949)
(*4*) Roe, J. H., and Papadopoulos, N. M., *J. Biol., Chem.*, **210**, 703 (1954)
(*5*) Imamoto, F., and Okunuki, K., *Kagaku no Ryoiki, Zokan*, **No. 20**, 39 (1955)
(*6*) Stein, M. W., Cori, G. T., and Cori, C. F., *J. Biol, Chem.*, **186**, 763 (1950)
(*7*) Kotake, Y. (Jr.), and Kato, M., *Proc. Japan Acad.*, **32**, 210 (1956), *Proc. Japan Acad.*, **32**, 361 (1956)
(*8*) Kotake, Y. (Jr.), and Nogami, K., *J. Biochem.*, **43**, 437 (1956)

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THE STRUCTURE OF CYTOCHROME C

I. THE C-TERMINAL RESIDUE OF CYTOCHROME C

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Cytochrome c has been extensively investigated chemically as well as physically by many workers, but the study of its chemical structure has only recently started. Margoliash (*1*) could detect 2 moles of histidine per mole protein as its N-terminal residue by DNP-method and concluded that cytochrome c consists of two peptide chains. Tuppy, Bodo and Paléus (*2, 3*) determined the amino acids sequence in the heme-peptides produced by the tryptic and peptic digestion of cytochrome c. Niu and Fraenkel-Conrat (*4*) found no C-terminal amino acids by the hydrazinolysis of cytochrome c.

In the present work, hydrazinolysis of cytochrome c was reinvestigated for the purpose of finding C-terminal amino dicarboxylic acids which might be lost in the experimental conditions used by Fraenkel-Conrat. Hydrazinolysate of cytochrome c, after freed from excess hydrazine, was subjected to paper-electrophoresis, whereby glutamic acid was clearly detected as shown in Fig. I. Results of semi-quantitative determinations by hydrazinolysis-DNP method (*5*) suggests that all three of cytochrome c of horse heart muscle, whale heart muscle and yeast bear 1 mole of C-terminal glutamic acid per mole protein.

EXPERIMENTAL AND RESULTS

Cytochrome c—Horse cytochrome c was prepared from minced heart by the method of Keilin and Hartree. In the case of whale initial extraction of the enzyme from the mince was carried out using dilute sulfuric acid in place of trichloroacetic acid. Yeast cytochrome c was

extracted by ammonium sulfate from autolysed yeast according to the method of Hagihara *et al.* (*6*). Each of the extracted cytochrome c was purified by using IRC-50 as ion exchange resin.

Hydrazinolysis—Hydrazinolysis was carried out according to the method of Akabori (*5*). 5 to 15 mg. of cytochrome c were hydrazinolysed with about 0.5 ml. of anhydrous hydrazine at 100° in a sealed tube. The time of hydrazinolysis was varied from 1 to 6 hours. The reaction mixture was evaporated *in vacuo* over sulfuric acid to remove excess hydrazine.

Electrophoresis—In the case of qualitative determination of terminal residue, the residue was dissolved in about 2 ml. of water and electrophoresed on Toyo filter paper No. 51 in lutidine-acetate buffer at pH

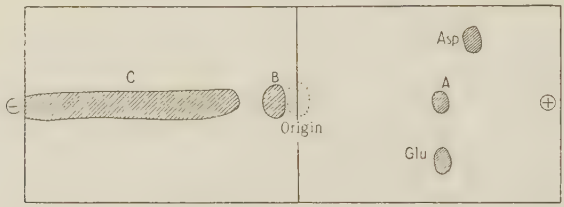

Fig. 1. Qualitative detection of glutamic acid as C-terminal amino acid of cytochrome c by a paper electrophoresis.

A : Detected glutamic acid. B : Mono-hydrazides of acidic amino acids. If C-terminal amino acid is neutral, it comes here. C : Hydrazides of amino acids. If C-terminal amino acid is basic, it comes in this portion.

The conditions of an electrophoresis are described in the text.

6.2 for 3 hours (20 volts per cm.) according to the method of Narita (*7*). The buffer was prepared by adding 36.3 ml. of lutidine and 185 ml. of 1 *N* acetic acid to 3.86 litre of water. The results are shown in Fig. 1. Glutamic acid was detected in every case.

Aldehyde Treatment and Dinitrophenylation—The following method was almost similar to the method of Niu and Fraenkel-Conrat (*4*), but enanthol was used in place of benzaldehyde (*8*). The residue freed from excess hydrazine was dissolved in 2 ml. of water and 0.5 ml. of enanthol was added. After shaking for 10 minutes, the solution was centrifuged to separate the layers of aldehyde and water completely. The upper aldehyde layer together with the condensation product of the hydrazides with enanthol was removed. 0.5 ml. of enanthol was

again added into the water layer. After shaking for one hour the aldehyde layer was removed after separation by centrifugation. The water layer was transferred to a test tube. The centrifugation tube and the separation funnel used were washed with 1 ml. of water twice. The washings were added to the test tube, to which 1 ml. of 5 per cent sodium bicarbonate solution, 5 ml. of ethanol and 1 ml. of 5 per cent fluorodinitro-

TABLE I

*Estimation of the C-terminal Glutamic Acid of Cytochrome c**

Origin	Time of hydrazinolysis (*hrs.*)	Cytochrome c		C-terminal glutamic acid		
		mg.	10^{-7} *M*	Found** 10^{-7} *M*	Glu/Cyt-c (uncorrected)	Glu/Cyt-c*** (corrected)
Horse	1	14.0	10.3	3.10	0.30	0.91
Horse	3	11.1	8.3	1.05	0.13	0.65
Horse	6	13.6	10.0	1.75	0.18	0.97
Yeast	6	5.0	3.7	0.65	0.18	0.97
Whale	6	10.8	7.9	1.18	0.15	0.80

* The molecular weight of cytochrome c is assumed to be 13,600.

** The molar extinction of DNP-glutamic acid at 360 mμ is taken to be 17.400.

*** Corrected by the factor cited in Table II.

TABLE II

Recovery of Glutamic Acid after "Hydrazinolysis"

Time of hydrazinolysis (*hrs.*)	Initial glutamic acid		Recovered glutamic acid	
	mg.	10^{-7} *M*	10^{-7} *M*	Recovery factor
1	1.39	9.45	3.18	0.33
3	1.39	9.45	1.92	0.20
6	1.39	9.45	1.76	0.19

benzene (DNFB) in ethanol were added. The dinitrophenylation was carried out at room temperature for 3 hours with shaking. The reaction mixture was diluted with 30 ml. of water, acidified with 6 *N* hydrochloric acid and extracted 3×10 ml. of ethyl acetate. The ethyl acetate solution was then diluted with two volumes of ether and extracted 4×10 ml. of 5 per cent sodium bicarbonate solution. The sodium bicarbonate

solution was acidified with 6 *N* hydrochloric acid and extracted 3 × 10 ml. of ethyl acetate. The solvent was removed *in vacuo* and dinitrophenol was eliminated by sublimation using Mill's apparatus.

Chromatographic Separation and Quantitative Estimation of C-terminal Amino Acid—The residue was dissolved in ethyl acetate and quantitatively transferred to Toyo filter paper No. 51 for two-dimensional paper chromatography. The first dimensional solvent was n-butanol saturated with 1 *N* ammonia water. The second was 1.5 *M* sodium phosphate buffer, pH 6.4. The spot of DNP-glutamic acid appeared clearly and the faint spots of DNP-serine, DNP-threonine, DNP-alanine, DNP-glycine and dinitrophenol were seen. In the case of whale cytochrome c, the additional spot of DNP-valine or DNP-leucine appeared feebly. After elution with 1 per cent sodium bicarbonate solution, the amount of DNP-amino acids in each spot was estimated spectrophotometrically. Readings of the optical density were taken at 360 mμ. The amounts of C-terminal glutamic acid are listed in Table I. The amounts of other DNP-amino acids were less than 0.2 mole per mole of protein, considering the recovery factor of each amino acid reported by Fraenkel-Conrat (*4*).

Control Experiments—To find a correction factor for the decomposition of glutamic acid, a weighed amount of glutamic acid was treated with anhydrous hydrazine under the same condition as used in the experiment of cytochrome c. The " hydrazinolysate " was treated in the same manner as described above, and recovery of glutamic acid was estimated quantatively. The recovery factor is shown in Table II.

DISCUSSION

From the results shown in Table I, it may be concluded that every cytochrome c has 1 mole of glutamic acid as its C-terminal residue per mole of protein. And it is interesting that glutamic acid is common to C-terminal of every cytochrome c in spite of the considerable differences between mammalian heart muscle and yeast cytochrome c (*9*, *10*).

SUMMARY

The C-terminal amino acid of horse heart muscle, yeast and whale heart muscle cytochrome c was tested by hydrazinolysis method, and 1 mole of glutamic acid was detected per 1 mole of every cytochrome c.

The authors wish to express their thanks to Prof. S. Akabori, Prof. H. Yoshi-

kawa, Prof. K. Satake and Prof. Y. Yoneyama for constant guidances in the course of the work. Thanks are also due to Dr. K. Narita and Mr. K. Kusama for their kind technical advices.

REFERENCES

(*1*) Margoliash, E., *Nature*, **175**, 293 (1955)
(*2*) Tuppy, H., and Bodo, G., *Monatsch.*, **85**, 1024 (1954)
(*3*) Tuppy, H., and Paléus, S., *Acta Chem. Scand.*, **9**, 353 (1955)
(*4*) Niu, C. I., and Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, **77**, 5882 (1955)
(*5*) Akabori, S., Ohno, K., and Narita, K., *Bull. Chem. Soc. Japan*, **25**, 214 (1952) ; Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, I., Tsugita, A., Sugae, K., and Matsushima, T., *Bull. Chem. Soc. Japan*, **29**, 506 (1956)
(*6*) Hagihara, B., Horio, T., Yamashita, J., Nozaki, M., and Okunuki, K., *Nature*, **178**, 629 (1956)
(*7*) Narita, K., and Kamata, Y., The 9th Ann. Meeting of Chem. Soc. Japan (Kyoto, April, 1956)
(*8*) Kusama, K., Symp. on Protein Structure (Fukuoka, Oct., 1956)
(*9*) Minakami, S., Titani, K. and Ishikura, H., Symp. on Protein Structure (Fukuoka, Oct., 1956)
(*10*) Titani, K., Ishikura, H., and Minakami, S., The 85th Kanto District Meeting of Japan Biochem. Soc. (Tokyo, Nov., 1956)

The Journal of Biochemistry, Vol. 44, No. 8, 1957

BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

IV. THE CONVERSION OF PYRUVIC ACID TO ALANINE IN THE SILKWORM LARVA

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(Received for publication, April 3, 1957)

Silkworms (*Bombyx mori*) eat mulberry leaves and spin their cocoons, but the content of each amino acid in the silkproteins (*1*) is strikingly different from that of mulberry leaves (*2*). In view of the high alanine content (25 per cent) of the cocoon fibres, Kirimura (*3*) compared quantitatively the alanine intake of the silkworms with the alanine content of the cocoon fibres produced, and indicated that alanine must be synthesized *in vivo*. Bheemeswar (*4*) indicated that alanine was synthesized from aspartic acid by β-decarboxylase present in the silkglands of the silkworms. Recently Florkin *et al.* (*5*) demonstrated, using radioactive phenylalanine-1-C^{14}, that the carboxyl carbon of phenylalanine was not utilized for the synthesis of alanine of the silk by silkworm. The present work was carried out to examine whether pyruvic acid is utilized for the synthesis of alanine in the silk by *Bombyx mori*.

EXPERIMENTAL AND RESULTS

The first experiment was planned to ascertain whether the isotope (C^{14}) of the sodium pyruvate-2-C^{14} given to the silkworms appears in the alanine isolated from the cocoon fibres produced by these silkworms. Radioactive sodium pyruvate-2-C^{14} (3.17 mc per mM) was obtained from the Radiochemical Centre, Amersham, England. 0.5 μc of radioactive sodium pyruvate (17.5 μg. as keto acid) per worm was given per os to the silkworms, Nichi 122×Si 115, at the fourth day of the fifth instar. These silkworms continued to feed normally for 72 hrs. and then began spinning their cocoons. Specific activity of the cocoon fibre measured with an SC-16 windowless gas-flow counter (Tracerlab. Inc.) was 3,443 c.p.m./100 mg. (average). 6 g. of the radioactive cocoon fibres was cut into small pieces, and degummed by boiling with distilled water and

M/50 sodium carbonate solution repeatedly, until the weight of the fibroin became constant: the yield was 4.28 g. 4 g. of the fibroin was hydrolysed by refluxing in 12 *N* hydrochloric acid for ten hours. From the hydrolysate, alanine was removed as a salt of azobenzene-*p*-sulphonate, and then alanine was separated from its salt, following the procedure described in the previous paper (*6*). The alanine was recrystallized from water by repeated addition of absolute ethanol until its specific activity became constant. The yield was 459 mg. (11.5 per cent of fibroin). The chemical purity of the purified alanine was proved by a paper chromatography. Radioactivity of this amino acid was 2,892 c.p.m./100 mg. The isolated alanine was analysed by a paper chromatography, using phenol containing 0.1 per cent ammonia as a solvent. The strip was sprayed with ninhydrin in the usual way, the position of alanine marked and the strip scanned for radioactivity by an SC-16 windowless gas-flow counter. High radioactivity was recog-

Table I

Distribution of C^{14} in Alanine Molecule

Position in molecule	*c.p.m./mg.*
Carboxyl (—COOH)	5
(—$CHNH_2$—)	80
(CH_3—)	2

nized at the position of alanine. A radioautograph obtained by superposition of an X-ray film for three weeks on the chromatogram of isolated alanine gave also an image at the position of alanine. Also, the isolated alanine was degraded stepwise according to the procedure described by Vernon *et al.* (*7*). It was found to have a higher labeling in the 2C position of the alanine molecule (Table I). These results would seem to suggest that the alanine isolated is labeled with isotopic carbon.

The second experiment was carried out to determine where the conversion of pyruvic acid to alanine takes place chiefly in the silkworm body. The silkglands, alimentary canals, muscle and fat tissues obtained from five larvae on the sixth day of 5th instar, Si 110×Nichi 122, were washed with cold water, suspended in *M*/15 phosphate buffer, pH 7.4, homogenized and made up to 5 ml. Each sample was placed for 5 hours at 0° and then kept frozen at −18° overnight. The insoluble proteins

obtained by thawing the ice at room temperature were discarded and the supernatant solution was used as an enzyme solution. To 1 ml. of each enzyme solution were added 0.5 ml. of 0.1 *M* L-glutamic acid, 0.5 ml. of 0.1 *M* sodium pyruvate and 0.1 ml. of *M*/15 phosphate buffer, pH 7.4, containing 0.1 μc of sodium pyruvate-2-C^{14}, and the mixture was incubated for 60 minutes at 38°. The solutions of L-glutamic acid and sodium pyruvate were freshly prepared by desolving in *M*/15 phos-

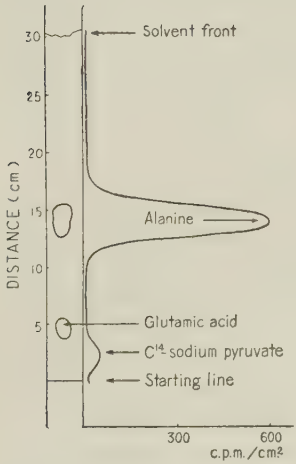

FIG. 1. Radioactivity of chromatogram of the reaction mixture.

The reaction mixture contained 1 ml. of the enzyme solution obtained from the posterior division of the silkglands, 0.5 ml. of 0.1 *M* L-glutamic acid, 0.5 ml. of 0.1 *M* sodium pyruvate and 0.1 ml. of *M*/15 phosphate buffer containing 0.1 μc of sodium pyruvate-2-C^{14}. Incubation time, 1 hour at 38° ; pH 7.4. Solvent system: *n*-butanol-acetic acid saturated with water.

TABLE II

Radioactivities of the Alanine Spot on the Chromatograms

Tissues	c.p.m. of alanine spot formed
Posterior division of silkgland	420
Alimentary cannal	330
Muscles and fat tissues	273

phate buffer, pH 7.4, just prior to use. The reaction was stopped by adding 200 mg. of trichloracetic acid. This filtrate was analysed by means of a paper chromatography, using *n*-butanol-acetic acid saturated with water (*n*-butanol 95 : acetic acid 5) as a solvent. Two spots were revealed on a strip which was sprayed with ninhydrin in the usual way : one of them (R_f value 0.42) was alanine formed and the other (R_f value 0.16) was glutamic acid remained in the reaction mixture. The strip

was scanned for radioactivity by an SC-16 windowless gas-flow counter. As shown in Fig. 1, a higher radioactivity was recognized at the position of the alanine. A lower radioactivity was also recognized at the position of R_f 0.14, at nearly the same position as glutamic acid, but this proved, by comparing with the behavior of pure sodium pyruvate-2-C^{14} on a chromatogram, to be due to the isotopic sodium pyruvate remained in the reaction mixture. A radioautograph obtained by superposition of an X-ray film on the chromatogram gave each image at the position of the alanine and sodium pyruvate. Radioactivities of the alanine spot on the chromatograms obtained from three tissues of the silkworm are presented in Table II. This result seems to suggest that the synthesis of alanine from pyruvic acid with glutamic acid takes place in all the silkglands, the alimentary cannal, muscle and fat tissues.

The third experiment was carried out to clarify what kind of amino acid plays the most important role in the synthesis of alanine from pyruvic acid. The following 14 amino acids were surveyed by adding 1 ml. of each amino acid solution to a mixture containing 1 ml. of the enzyme solution prepared from the silkglands and 0.5 ml. of the sodium pyruvate solution, and incubating the mixture for 60 minutes at 38° as mentioned above: L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-leucine, DL-methionine, L-phenylalanine, DL-serine, DL-threonine, L-tryptophan, L-tyrosine and DL-valine. Solutions of the amino acids and keto acid were freshly prepared, prior to use, with 0.05 *M* phosphate buffer, pH 7.4, at a concentration of 50 μM per ml. with respect to the L-form and 200 μM per ml. The enzyme solution was prepared, as described above, from the posterior division of the silkglands on the sixth day of the 5th instar, Nichi 122 × Si 115. The concentration of the alanine formed in the reaction mixture was estimated with the procedure described by Giri (*8*, *9*). A summary of the results is presented in Table III, which shows an approximate amount of transamination as expressed in micromoles of alanine formed per 1 g. of the fresh tissues. It is especially of note that the most active transaminases are those involving glutamic and aspartic acid.

DISCUSSION

In the present work, it was elucidated, using isotopic sodium pyruvate, that alanine was synthesized from pyruvic acid in the presence of amino acids, especially glutamic acid or aspartic acid in all the silkglands, the alimentary cannal, the muscles and the fat tissues of the silkworm

larva. Recently the occurrence of the transamination reaction in the silkworm was also indicated by Bheemeswar *et al.* (*10*, *11*) and Koide *et al.* (*12*). As glutamic acid and aspartic acid are the main constituent amino acids of the proteins of the mulberry leaves, this reaction which produces alanine from pyruvic acid in the presence of glutamic acid or aspartic acid seems to be of special importance in the synthesis of the alanine of the silk, besides the alanine formation by β-decarboxylase from aspartic acid in the silkworm (*4*).

In the previous paper (*13*), the author and the co-workers reported the attempted isolation of keto acids of physiological importance in the body fluid and the silkglands of the silkworm larvae and clarified that

TABLE III

Transaminase Activity of the Silkglands

Amino acid	Micromoles of alanine formed
Arginine	15.0
Aspartic acid	54.0
Gystine	19.0
Glutamic acid	83.0
Glycine	14.0
Histidine	14.0
Leucine	15.0
Metyionine	9.0
Phenylalanine	17.0
Serine	16.0
Threonine	18.0
Tryptophan	18.0
Tyrosine	15.0
Valine	19.0

there were glyoxylic, α-ketoglutaric, oxalacetic and acetoacetic acid in the silkworms. On this occasion, pyruvic acid found usually in the mammals was not recognized in the silkworms. However, the present work demonstrated that, although pyruvic acid was not found in the body fluid and the silkglands, the metabolism involving pyruvic acid takes place in the silkworm.

SUMMARY

1. The present work was carried out, using sodium pyruvate-2-C^{14}, to examine whether pyruvic acid is utilized for the synthesis of alanine in the silk by *Bombyx mori*.

2. The isotope (C^{14}) of the sodium pyruvate-2-C^{14} given to the silkworms appeared in the 2C position of the alanine isolated from the cocoon fibres produced by these silkworms.

3. Alanine was synthesized from pyruvic acid in the presence of amino acids, especially glutamic acid or aspartic acid in all the silk-glands, the alimentary cannal, the muscles and the fat tissues of the silkworm larvae.

4. These facts seem to suggest that the conversion of pyruvic acid to alanine in the silkworm larva is of special importance in the synthesis of the alanine of the silk.

REFERENCES

(*1*) Fukuda, T., Kirimura, J., Matuda, M., and Suzuki, T., *J. Biochem.* (*Japan*), **42**, 341 (1955)
(*2*) Kirimura, J., unpublished.
(*3*) Kirimura, J., unpublished.
(*4*) Bheemeswar, B., *Nature*, **176**, 555 (1955)
(*5*) Bricteux-Gregoire, S., Verly, W. G., and Florkin, M., *Nature*, **177**, 1237 (1956)
(*6*) Fukuda, T., *Nature*, **177**, 429 (1956) ; *J. Biochem.* (*Japan*), **43**, 137 (1956)
(*7*) Vernon, L. P., and Arronoff, S., *Arch. Biochem.*, **29**, 179 (1950)
(*8*) Giri, K. V., and Rao, N. A. N., *Nature*, **169**, 923 (1952)
(*9*) Giri, K. V., Radhakrishnan, A. N., and Vaidyanthan, C. S., *Nature*, **170**, 1025 (1952)
(*10*) Bheemeswar, B., and Sreeninasaya, M., *Current Sci.* (*India*), **21**, 253 (1952)
(*11*) Bheemeswar, B., and Sreeninasaya, M., *J. Sci. Ind. Research* (*India*), **13 B**, 108 (1954)
(*12*) Koide, F., Nagayama, H., and Shimura, K., *J. Agr. Chem. Soc. Japan*, **29**, 987 (1955)
(*13*) Fukuda, T., Hayashi, T., and Matuda, M., *J. Japanese Biochem. Soc.*, **27**, 147 (1955)

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LIPID MATERIAL OF BACILLUS ALCALIGENES FAECALIS

I. STUDIES ON THE ACETONE INSOLUBLE FRACTION* BY PAPER ELECTROPHORESIS

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(Received for Publication, April 5, 1957)

In the earlier studies made by one of the authors to elucidate the chemical composition of microorganisms analyses were made for lipid materials isolated from several pathogenic and non-pathogenic bacteria (*1–8*). This paper deals with the acetone insoluble fraction especially, phosphoglycerides of *B. alcaligenes faecalis*. The investigation was carried out chiefly employing the techniques for paper electrophoresis with the results that the phosphoglycerides were separated into two fractions, *i.e.*, phosphatidic acids and phosphoglycerides, of which the latter containing ethanolamine and polypeptides as constituents. A detailed description is given in the following.

EXPERIMENTAL AND RESULTS

Extraction of Lipids from B. alcaligenes faecalis—The bacterial cells were cultured in ordinary broth for 5 days, the moist bacteria harvested were spread on glass plates in a thin layer and dried at 37°. 450 g. of dry thin sheets of bacterial cells thus obtained were thoroughly disintegrated in a stainless steel ball mill and were extracted three times with alcohol-ether (1:1) at room temperature. From the combined extracts were obtained the lipids as a deep brown colored greasy matter which weighed 11.31 g., corresponding to 2.51 per cent on the dry weight basis of bacteria.

Fractionation of the Lipids into Acetone Soluble and Insoluble Fractions—The fractionation was carried out by repeating three times the procedure —the dissolution of the entire lipids in 15 ml. of ether and precipitation

* This work was announced at the 30th Anniversary Meeting of the Japanese Biochemical Society held in Tokyo on November 2, 1955.

by the addition of four volumes of acetone with ice cooling. The final precipitate representing the acetone insoluble fraction formed a light brown soft solid amounting to 4.41 g. (39.0 per cent). The acetone soluble fraction obtained by evaporating the combined supernatants gave a dark greasy substance, weighing 5.31 g. (47.0 per cent). The research made on the acetone soluble fraction will be published in a subsequent report.

Separation of the "Crude" Phosphoglycerides from the Acetone Insoluble Fraction—4.41 g. of the acetone insoluble fraction were suspended in 80 ml. of petroleum ether (30°–60°), thoroughly mixed and kept in the ice box overnight, after which it was centrifuged. The precipitate was reserved as sphingolipid fraction for future study. The supernatant fluid was distilled *in vacuo* and the resulting residue was dissolved in ether, to which 4 volumes of ice cold acetone were added. After keeping the mixture in the ice box overnight, the precipitated crude phosphoglyceride fraction was separated by centrifugation and washed three times with ice cold acetone-ether (4:1). A light brown, hygroscopic solid was obtained, which weighed 3.70 g. (84 per cent).

Analysis for the "Crude" Phosphoglycerides—P ; 3.75 per cent, N ; 1.5 per cent, P :N=1 :1.21. Ten μl. aliquots of 1 per cent solution of sample in chloroform equivalent to 100 μg. of sample gave the ninhydrin reaction, the modified Hanes-Isherwood reaction, and the phosphomolybdic acid reaction, but not the aniline hydrogen phthalate reaction. The reaction procedures are stated in the following.

(*a*) *Ninhydrin Method*—The strip spotted with sample was sprayed with 0.1 per cent ninhydrin in water saturated *n*-butanol and heated at 105° for 5 minutes.

(*b*) *Modified Hanes-Isherwood Method*—The modified spray reagent is the admixture made by mixing 2 ml. of 70 per cent $HClO_4$, 5 ml. of 8.3 per cent ammonium molybdic acid, 2 ml. of concentrated HCl, 40 ml. of chloroform, 20 ml. of ether, 20 ml. of methanol and 25 ml. of ethanol. The paper to be tested was sprayed with the above reagent, heated at 50° for 3 minutes, and then irradiated for a few minutes by ultraviolet light, when a well defined blue colored spot was revealed.

(*c*) *Phosphomolybdic Acid Method*—The paper to be visualized was dipped into a 5 per cent aqueous solution of phosphomolybdic acid for 1 minute for choline or several minutes for lecithin, washed by immersion in *n*-butanol for 5 minutes and then in running tap water until the colored paper turned colorless, with a light yellow spot remaining on it. Finally, the paper was passed through a freshly prepared solution of 0.4 per cent

stannous chloride in 3 *N* HCl. This reagent is able to locate a trace amount of lecithin (8 μg.), as well as free organic bases such as choline (6 μg.) and trimethylamine (10 μg.). Ethanolamine does not react at all with this reagent while methylamine reacts with it to form yellow spots, which soon disappear by further soaking in the reagent.

(*d*) *Aniline Hydrogen Phthalate Method*—This was conducted in an ordinary manner.

Paper Electrophoresis of a Synthetic Mixture of Phosphoglycerides in an Organic Buffer Medium—This study was carried out by using the technique introduced by Kanngiesser (*9*). Samples used were phosphatidic

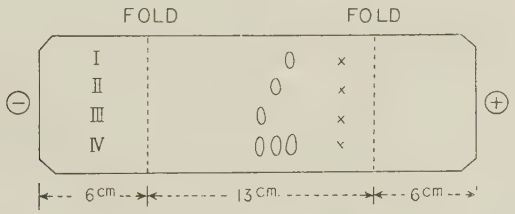

Fig. 1. Paper electrophoresis of a synthetic mixture of phosphoglycerides in an organic buffer medium.

Sample applied : 100μg. of each sample dissolved in 10 μl. chloroform.

I : Phosphatidic acids isolated from *B. natto*.

II : Brain cephalin fraction.

III : Brain lecithin.

IV : Synthetic mixture containing 100 μg. each of the above three samples.

Spray reagent, the modified Hanes-Isherwood's reagent.

acids from *B. natto*, brain cephalin fraction, and brain lecithin, all of which were isolated by the authers. A strip of Tôyô filter paper No. 51, 7 cm. wide and 25 cm. long, was soaked in a solvent mixture, glacial acetic acid-chloroform-pyridin (1 :1 :1 by volume). The resolution of the individual spots took place in 12 hours with a field strength of 28 volts per cm. in an organic buffer at pH 7.0 mentioned above. After completion of the electrophoresis, spots were obtained by developing with the modified Hanes-Isherwood reagent, and they were shown in Fig. 1. This makes it clear that a synthetic mixture of phosphatidic acids, cephalin fraction, and lecithin are separable from each other under conditions described above.

Paper Electrophoresis of the "Crude" Phosphoglycerides in an Organic Buffer Medium—100 μg. of the crude phosphoglycerides from the acetone insoluble fraction were dissolved in 10 μl. of chloroform and subjected to paper electrophoresis in the same manner as mentioned above. After the electrophoresis was over, the dried strip was cut lengthwise into three small strips of equal size. These strips were treated for the ninhydrin reaction, the modified Hanes-Isherwood reaction, and the phosphomolybdic acid reaction, all of which were positive. In practicing

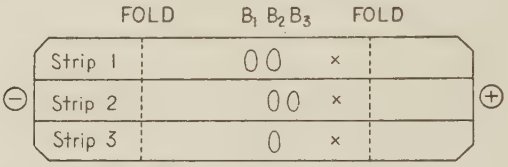

Fig. 2. Paper electrophoresis of the "crude" phosphoglycerides in an organic solvent mixture.
Strip 1 : Ninhydrin method.
Strip 2 : Modified Hanes-Isherwood method.
Strip 3 : Phosphomolybdic acid method.

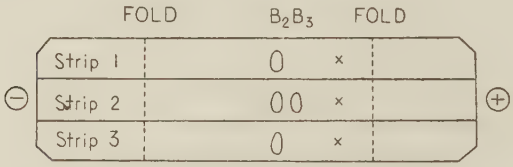

Fig. 3. Paper electrophoresis of the "purified" phosphoglycerides in an organic solvent mixture.
Strip 1 : Ninhydrin method.
Strip 2 : Modified Hanes-Isherwood method.
Strip 3 : Phosphomolybdic acid method.

the phosphomolybdic acid reaction care was taken so that the strip was previously washed in running tap water for 5 minutes before being dipped into the reagent, otherwise it was difficult to obtain a well contrasted pattern with colorless background. The three colored strips 1, 2, and 3, were brought together so as to recover the original shape and the whole results obtained were examined, which are illustrated in Fig. 2.

The figure indicates that the crude phosphoglycerides are separable into three subfractions, B_1, B_2, and B_3. From this can be suggested that

B_1 contains substances which give merely the ninhydrin reaction, B_2 is a fraction which reveals all of the three reactions above mentioned, and B_3 includes substances which contain phosphates in organic combination. It is noticeable that B_2 and B_3 of the three fractions are soluble in ether, while B_1 is soluble in water. In this connection, the question arises as to whether this B_1, the water soluble and ninhydrin positive fraction, be due to coloration by an essential component of the phosphoglycerides or by some contaminants from other sources.

Paper Electrophoresis of the So-called "Purified" Phosphoglycerides—In order to answer the above question, the crude phosphoglycerides were submitted to the purification procedure by electrodialysis at 700 volts through cellophane membranes. The electrodialyzed or so-called "purified" phosphoglycerides which were analyzed gave P 5.64 per cent, N 2.18 per cent, and P:N=1:0.85. The electrophoretic study on the purified phosphoglycerides which was undertaken under conditions identical with the crude phosphoglycerides demonstrated the presence of B_2 and B_3 bands and the absence of B_1 band in the purified sample examined as represented in Fig. 3. This result indicates that B_1 may be due to some contaminants removable by electrodialysis and may not be the components of the phosphoglycerides proper.

Hot Acetone Soluble and Insoluble Fractions of the Purified Phosphoglycerides Examined by Electrophoresis—100 mg. of the purified phosphoglycerides were refluxed with 10 ml. of acetone for 1 hour. The mixture was filtered hot through a sintered glass filter, and the insoluble fraction separated was treated with hot acetone in a similar manner twice more. The combined filtrates, which were somewhat turbid and colored pale yellow, yielded upon vacuum evaporation 25 mg. of hot acetone soluble fraction as a brown paste, corresponding to 25 per cent by weight of the purified phosphoglycerides employed. The fraction insoluble in hot acetone gave a light brown solid. Samples from both fractions were dissolved in chloroform and examined by electrophoresis. The result obtained showed that both were similar to the original purified phosphoglycerides in so far as they were composed of B_2 and B_3 subfractions. Since hot acetone treatment seemed ineffective for fractioning, the following analyses were conducted with the purified sample, *i.e.*, the cold acetone insoluble and electrodialyzed phosphoglyceride fraction.

Total Hydrolysis of the Purified Phosphoglycerides—5 mg. of sample together with 2 ml. of 5 per cent HCl in methanol were sealed in a small glass tube and heated in a boiling water-bath for 12 hours, after which the hydrolysate was extracted with an equal volume of ether several

times repeatedly. Thus the hydrolysate was separated into two fractions, an ether soluble and a water soluble ones.

Fatty Acids—Evaporation of the ether extract gave a greasy substance, which was subjected to saponification. An ether solution of the saponified material was applied to a paper to form a spot, and a distinct blue spot of copper soap was obtained when the paper was dipped into a dilute copper acetate solution.

Water Soluble Hydrolysis Products—The aqueous layer was transferred into a watch glass and vacuum evaporated to dryness repeatedly with water in a desiccator over NaOH to eliminate the excess HCl. The final dry residue was dissolved in 0.25 ml. of water. With 10 μl. aliquots of this solution representing 200 μg. of the original matrial gave positive

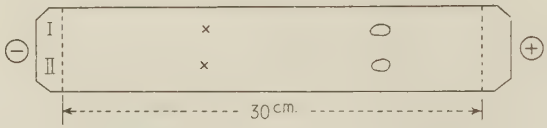

Fig. 4. Electrophoresis of the hydrolysate of the purified phosphoglycerides showing the presence of α-glycerophosphoric acid.

I : α-glycerophosphoric acid.

II : Sample of the hydrolysate.

× : Site of application.

Sample amount, 5 μl. of the hydrolysate corresponding to 100 μg. of purified phosphoglycerides. Paper, Toyo Paper No. 51, 6×35 cm. ; Field strength, 10.0 volts per cm. ; Duration, 2 hours ; Operation at room temperature ; Spotting, the modified Hane-Isherwood method as well as the ammoniacal silber nitrate method.

tests for the ninhydrin reaction, the modified Hanes-Isherwood reaction, and the ammoniacal silber nitrate reaction, while the phosphomolybdic acid reaction and the aniline hydrogen phthalate reaction were all negative. The following substances were detected, but there seemed to be some other unidentified substances in this hydrolysate.

α-Glycerophosphoric Acid—Identification of this substance was made by both paper chromatography and electrophoresis, as shown below.

The paper chromatograms which were run with 5 μl. aliquots of the sample solution revealed a spot of R_f value 0.49 in *n*-butanol-acetic acid-water (4 :1 :5) and a spot of R_f value 0.72 in *n*-propanol-conc. ammonia-water (6 :3 :1), both showing an identity with those of α-glycerophosphoric acid. There was no indication of the presence of β-

glycerophosphoric acid which run simultaneously as control and gave much smaller R_f values (0.07, and 0.18) than α-type.

Separation and Identification of Individual Ninhydrin Positive Substances— The occurrence of lysine, glutamic acid, neutral amino acids (glycine and serine), ethanolamine, an unidentified base and an unknown ultraviolet absorbing substance in the hydrolysate, was demonstrated by electrophoresis, the results of which are illustrated in Fig. 5.

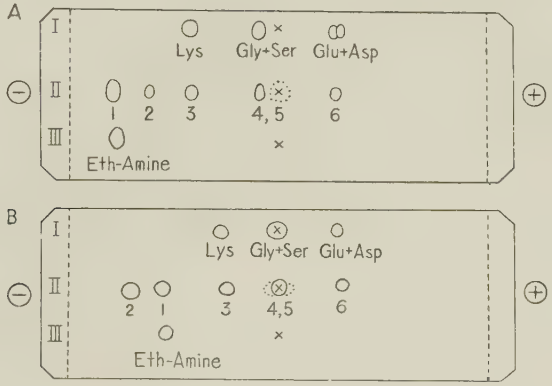

FIG. 5. Electrophoretic patterns of the hydrolysate of the purified phosphoglycerides.

A : Phosphate buffer (pH 7.0, ionic strength 0.16).

B : Acetate buffer (pH 6.2, ionic strength 0.16)

I : Standard amino acid mitxure comprising lysine, glycine, serine, glutamic acid and aspartic acid.

II : Sample of the hydrolysate.

II : Ethanolamine.

× : Site of application.

Sample applied, 5 μl. of the hydrolysate corresponding to 100 μg. of the purified phosphoglycerides ; Paper, Toyo Paper No. 51, 9×35 cm. ; Field strength, 10.0 volts per cm. ; Duration of electrophoresis, 1.5 hour ; Operation at room temperature ; Spray reagent, 0.1 per cent ninhydrin in water saturated *n*-butanol.

As indicated in these patterns, the hydrolysate was separated into five ninhydrin positive spots 1, 2, 3, 4, 6 and one ultraviolet absorbing spot 5. Among five ninhydrin positive spots, three spots 1, 2, and 3 were basic, spot 4 was neutral and spot 6 was acidic in nature. By comparing with the reference samples run simultaneously (I), it is likely to be considered that among three basic spots, 1, 2, and 3 in both A and

B patterns, spot 1 was ethanolamine, and spot 3 lysine, while spot 6 on the acid side was glutamic acid.

Further confirmation by paper chromatography of this finding was conducted as follows.

(*a*) *Lysine*—Since lysine is difficultly separable from arginine by paper electrophoresis in neutral buffer, the lysine portion (spot 3) of the chromatogram located by a control pattern was cut off and extracted with 0.001 *N* HCl and the extract was vacuum evaporated to dryness repeatedly with water in a desiccator over NaOH to remove the excess HCl. The evaporation residue was dissolved in a small amount of water and analyzed by paper chromatography, which showed a spot of R_f 0.2 in *n*-butanol-acetic acid-water (4 :1 :5) and a spot of R_f 0.63 in a system 1 :1 *m*-cresol-phenol-pH 8.4 borate buffer. The latter chromatography was conducted on the paper impregnated with pH 8.4 borate buffer, according to the procedure improved by Levy and Chung (*10*). The values obtained were quite in accord with those of an authentic sample of lysine.

(*b*) *Glutamic Acid*—This substance did not need further identification, because glutamic acid was able to be differentiated with ease from aspartic acid on the chromatogram by the migration rate as well as the color tone developed with ninhydrin.

(*c*) *Neutral Amino Acids* (*Glycine and Serine*)—The neutral amino acid portions (spot 4) cut off from five sheets of chromatograms were combined together and extracted with 0.001 *N* KOH and the extract which was desalted by Amberlite IR-120 and Amberlite IRA-410 was distilled *in vacuo* to dryness. The residue was dissolved in a suitable amount of water and chromatographed with a solvent mixture of 1 :1 *m*-cresol-phenol-pH 8.4 borate buffer in a manner similar to that described for lysine. Two single spots were revealed, one with R_f value of 0.18, corresponding to serine and another with R_f value of 0.26, indentical with glycine. Reference samples of alanine and threonine gave somewhat higher R_f values.

(*d*) *Ethanolamine*—The extract from spot 1 in A or B was examined by the ascending chromatography, which revealed equally a spot of R_f value of 0.73 in *n*-butanol-acetic acid-water (4 :1 :5) and a spot of R_f value of 0.75 in *n*-propanol-ammonia-water (6 :1 :3), both being identical with that of ethanolamine.

(*e*) *Unidentified Base*—As indicated in Fig. 3, spot 2 in both A and B is due to a substance which has a lower mobility than ethanolamine in phosphate buffer at pH 7.0, but a higher mobility in acetate buffer.

This inversion of mobility depending upon the type of buffer solution was also observed in the case of putrescin as well as agmatin, although neighter of them could not claim an identity with this substance. Elucidation of chemical nature of this substance needs further investigation with enough amounts of sample, if available.

(*f*) *Ultraviolet Absorbing Substance*—The ultraviolet absorbing spot 5 in both A and B was located almost immobile on the original line and very close to the neutral fraction. On the other hand this substance was revealed as a distinct ultraviolet absorbing spot with an R_f value of 0.30 on the paper chromatographic pattern which was obtained by using 5 μl. of the total hydrolysate above mentioned and a solvent system of *n*-butanol-acetic acid-water. The spot, however, showed none of these tests: the ninhydrin test, the modified Hanes-Isherwood test, and the aniline hydrogen phthalate test. The portion corresponding to the spot of the non-sprayed paper was extracted with 0.1 *N* HCl and the extract was examined by spectrophotometer giving $\lambda_{max.}=255.5$ and $\lambda_{min.}=227.5$. Because of its insufficient amount, this substance failed to be studied further, although the crude phosphoglycerides seemed to have contained a noticeable quantity of this substance, which, however, was lost to a great extent during the purification procedure through electrodialysis.

Isolation of Peptides by Partial Alkaline Hydrolysis of the Purified Phosphoglycerides—5 mg. of the purified phosphoglycerides were placed in a small tube and heated with addition of 0. 1 ml. of ethanol to form a clear solution, to which was then added 1 ml. of *N* KOH. After sealing, the tube was heated in a boiling water-bath for 15 minutes. The resulting hydrolysate was made acid with HCl and extracted with ether. The aqueous layer obtained was vacuum evaporated to dryness repeatedly with water to remove the excess HCl, and the residue was extracted three times with 90 per cent ethanol. The combined alcohol extracts were evaporated to dryness, and the final residue dissolved in a small amount of water was submitted to electrophoresis in acetate buffer at pH 6.0, as a result of which the separation of acidic, neutral, and basic fractions was performed. The similar results were obtained irrespective of the duration of alkaline hydrolysis from 15 to 45 minutes. The basic fraction was ethanolamine, while the acidic and the neutral fractions proved to be polypeptides as follows. The portions of the acidic and the neutral fractions were cut off from the five sheets of chromatogram run simultaneously and five cuts from the similar portion were combined together and extracted with 20 ml. of 0.01 *N* KOH at room temperature

overnight. After neutralization with HCl the extract was vacuum evaporated and the residue was hydrolyzed with 6 *N* HCl in a sealed tube by heating in a boiling water-bath for 20 hours. The hydrolysate was dried in a vacuum desiccator over NaOH and analyzed by paper electrophoresis in the manner described above. The ninhydrin treated chromatograms showed that the samples from the acidic as well as the neutral portion equally yielded glutamic acid, neutral mino acids and ethanolamine but no lysine. This result made it clear that the two different ninhydrin posive fractions resulting from mild alkaline hydrolysis, of which one being acidic and the other neutral, involve polypeptides containing ethanolamine besides amino acids as constituent. Although lysine was found in total hydrolysis products of the purified phosphoglyceride fraction, it was missing in those polypeptide fractions.

Characterization of B_2 and B_3 Fraction Obtained by Electrophoresis from the Purified Phosphoglycerides and of B_1 Fraction from the Crude Phosphoglycerides—As descrived above, the crude phosphoglycerides, if electrophoretically examined, proved to consist of three components B_1, B_2, and B_3, while the purified phosphoglycerides consist of two components, B_2 and B_3. In order to elucidate the chemical nature of these fractions, the extracts from the spots were subjected to electrophoretical studies as follows.

B_2 and B_3—Some 20 ml. of the purified phosphoglycerides were dissolved in chloroform and a small amount of the solution was subjected to paper electrophoresis. After completion of the electrophoresis, the portions on the paper strips assigned for B_2 and B_3 bands were cut off, and each cut of the similar portions was combined and extracted with 2×30 ml. of ether at room temperature for 24 hours. The ether extracts from B_2 and B_3 portions both yielded upon evaporation small quantities of graywhite waxy matters, which were electrophoretically reaffirmed to be homogeneous. Upon hydrolysis, B_3 yielded fatty acids and α-glycerophosphoric acid, but no bases, whereas B_2 gave rise to fatty acids, α-glycerophosphoric acid and various amino acids, as well as ethanolamine just as those found in the total hydrolysis of the purified phosphoglycerides. Thus, it is apparent that the purified phosphoglyceride fraction includes two subfractions B_2 and B_3, of which the latter is merely phosphatidic acids and the former is phosphoglycerides involoving ethanolamine and polypeptides.

B_1—B_1 portions cut off from the patterns were extracted with 0.01 *N* HCl for 24 hours at room temperature and dried in a desiccator under normal pressure. Aqueous solution of the residue was directly subjected to electrophoresis, and glutamic acid, lysine, neutral amino acids,

ethanolamine, and volatile amines such as methylamine, trimethylamine were identified.

Proof for the Absence of Choline in the Crude Phosphoglyceride Fraction—2.5 mg. of the crude phosphoglyceride fraction was hydrolyzed and the entire water soluble hydrolysis products was applied to a paper for chromatography. The phosphomolybdic acid-$SnCl_2$ reaction was negative. As the detection limit of this reagent for choline is approximately 6 μg., the original lipids can be considered to be practically free of choline.

DISCUSSION

As preliminary experiment a paper electrophoretic analysis of phospholipids was undertaken chiefly employing the method introduced by Kanngiesser. Based on this method, it was demonstrated that phospholipids, which behave in general quite immobile in aqueous medium, are able to migrate in a certain organic solvent system. A series of experiments performed in this study on a variety of synthetic mixtures of phospholipids indicated that a mixture of phosphatidic acids, cephalin fraction and lecithin were separated into its component lipids even though one of the components was present in amounts as little as 5 per cent. However, it is as yet unsuccessful by this method to subfractionate the cephalin fraction into phosphatidyl serine, phosphatidyl ethanolamine, diphosphoinositide. In view of this it must be borne in mind that the data obtained from paper electrophoresis of phospholipids in an organic solvent system do not necessarily account for the homogeneity of the individual lipids separated and also that the migration rate of lipids in organic solvent systems is generally quite reduced partly due to the diminished ionization of compounds in that media. Anyway, it is a remarkable fact that the crude phosphoglycerides from *B. alcaligenes faecalis* were able to be divided into three fractions B_1, B_2, and B_3 by paper electrophoresis in an organic solvent medium, of which B_1 was soluble in water, and B_2 and B_3 were soluble in ether. In case the electrophoresis of the lipids was conducted in an aqueous medium, they showed no mobilities at all regardless of the site of sample application and the strength of the electric field, so that even the water soluble B_1 fraction could not be separated from the ether soluble B_2 and B_3 fraction.

With regard to the purification of phospholipids, the fundamental problem is to eliminate the two types of contaminants from the lipids. One pype of impurities involves those lipids, the segregation of which

was incomplete by the conventional procedures of solvent-solvent fractionation, and the other type of impurities resulted from water soluble organic and inorganic materials which were dissolved in organic solvent containing phospholipids and were carried down by either adsorption or intermolecular salt formation when the phospholipids were precipitated.

As one step of purifying the crude phosphoglycerides, it was subjected to electrodialysis, for the purpose of removing the water soluble impurities. The so-called purified phosphoglycerides thus obtained gave 5.64 per cent P, 2.18 per cent N, and P :N ratio of 1 :0.85. As compared with the crude phosphoglycerides, the purified material possessed higher values for P and especially for N. Its somewhat lower value of P :N ratio as a monoamino-monophospholipid seems to be due to an appreciable amount of phosphatidic acids present in it. The purified phosphoglycerides were investigated by paper electrophoresis in an organic solvent system, and two bands, B_2 and B_3, were observed likewise, but no B_1 band.

Furthermore paper electrophoretic studies were made on B_1 fraction, which was the only water soluble fraction of the crude phosphoglycerides. The result was that B_1 included a mixture comprising various amino acids such as glutamic acid and lysine, neutral amino acids and volatile amines such as ethanolamine, methylamine and trimethylamine. B_1 fraction might be therefore considered as a mixture of substances which was loosely bound to the crude phosphoglycerides and was eliminated by electrodialysis. However, no exact information is available concerning their origin.

It has been experimentally evidenced that the purified phosphoglycerides consist of phosphatidic acids (B_3) and phosphoglycerides containing ethanolamine and polypeptides (B_2). In view of the fact that the fatty acid ester linkages and the phosphate ester bonds can be cleaved rapidly by certain enzymes of the bactera, and also that the bacteria used was mostly dead cells, owing to the prolonged culture extending some 5 days, it would appear likely to consider that the great amount of phosphatidic acids found in the bacterial lipids resulted mainly from the postmortem products. The most interesting result is the occurrence of the phosphoglycerides chemically associated with ethanolamine and polypeptides, but there is no information on its details now, and a further investigation will make it clear.

The phosphomolybdic acid method was first applied for the detection of choline in the hydrolysate of lecithin by Chargaff, Levene,

and Green (*11*). The present research has evidenced that by using this method, we were able to visualize not only minute amounts of water soluble organic bases such as choline and trimethylamine excepting ethanolamine and methylamine, but also to detect a trace amount of lipid materials such as ovolecithin (8 μg.) and the alcaligenes phosphoglycerides (20 μg.) without prior hydrolysis. Of the purified phosphoglycerides consisting of B_2 and B_3 fractions, B_2 fraction alone gives a positive reaction with phosphomolybdic acid, even though it contains none of these substances such as choline, trimethylamine and other bases which react with this reagent. Therefore, what component of B_2 may give this color reaction has not yet been determined.

The original Hanes-Isherwood method is capable of detecting water soluble both organic and inorganic phosphate compounds, whereas in the modified method, the prescription of this reagent was somewhat changed so as to make possible to detect the water soluble phosphoate compounds, as well as several phosphoglycerides such as lecithin, cephalin, saturated lecithin, and also sphingolipids. In addition, this method has an advantage of keeping the background colorless for one week whithout turning blue, while in the original method, the background is liable to coloration usually within some 6 hours.

SUMMARY

1. The electrophoretic separation of phosphoglycerides in an organic buffer system was described, which was able to separate a trace amount of a synthetic mixture of phosphatidic acids, cephalin fraction and lecithin into its component lipids.

2. The acetone insoluble fraction of the lipids from *B. alcaligenes faecalis* was analyzed in the same manner and it was fractionated into two parts, of which one was phosphatidic acids and the other phosphoglycerides containing ethanolamine and polypeptides in a bound form.

REFERENCES

(*1*) Akashi, S., and Tanigami, K., *Rep. Inst. Chem. Res. Kyoto Univ.*, **8**, 33 (1933)
(*2*) Akashi, S., *J. Biochem.*, **29**, 13 (1939)
(*3*) Akashi, S., and Tanigami, K., *Rep. Inst. Chem. Res. Kyoto Univ.*, **10**, 117 (1939)
(*4*) Akashi, S., and Itami, F., *Ibid.*, **11**, 197 (1941)
(*5*) Akashi, S., and Itami, F., *Ibid.*, **12**, 195 (1941)
(*6*) Akashi, S., and Itami, F., *Ibid.*, **13**, 1 (1944)

(7) Akashi, S., *Nippon Seikagaku Kaishi*, **17**, 173 (1943)
(8) Akashi, S., *Ibid.*, **18**, 303 (1944)
(9) Kanngiesser, W., *Biochem. Z.*, **325**, 12 (1953)
(10) Levy, A. L., and Chung, D., *Anal. Chem.*, **25**, 396 (1953)
(11) Chargaff, E., Levene, C., and Green, C., *J. Biol. Chem.*, **175**, 67 (1948)

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DECOMPOSITION OF HEMATIN IN THE REACTION SYSTEM OF DICYANHEMATIN-ASCORBIC ACID-HYDROGEN PEROXIDE

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There have been many reports dealing with the in vitro model reaction for the physiological break down of hemoglobin (*1–8*). Among these, the reaction system of hemoglobin-ascorbic acid-oxygen leading to the formation of verdohemoglobin may be of particular interest as one of the most useful model reactions for the physiological process.

In this reaction system, however, when the reaction was carried out in the presence of KCN, some green compounds are formed closely resembling to pseudohemoglobin. It has not yet been established that any of these green compounds is a single chemical entity nor their chemical constitution is elucidated.

Several reports have been appeared dealing with the similar reaction starting from dicyanhematin, the reaction product of which has so far not yet fully been investigated in respect with its chemical relation to pseudohemoglobin-heme.

The present authors intended to study the reaction of heme-decomposition starting from dicyanhematin in the presence of ascorbic acid reacting with hydrogen peroxide or oxygen more in detail. From the mentioned system, the authors could obtain some intermediates which have so far never been described, the properties of which will be reported here.

EXPERIMENTAL

Recrystallized hemin prepared by the method of Willstätter was dissolved in 10 ml. of $N/10$ NaOH solution and 90 ml. of 0.6 M KCN was added to it. The final heme concentration was made to 5×10^{-5} M. The optical density was measured by a photoelectric spectrophotometer of Hitachi Co. Most of the reactions took place in the absorption vessel.

RESULTS

I. Dicyanhematin-Ascorbic Acid-H_2O_2 System

(*A*) In this reaction system under the condition as indicated in Fig. 1, the color of the reaction solution turned to the green. At various time intervals, the reaction solution was reduced with dithionite, covered with liquid paraffin to avoid the contact of air and the absorption was

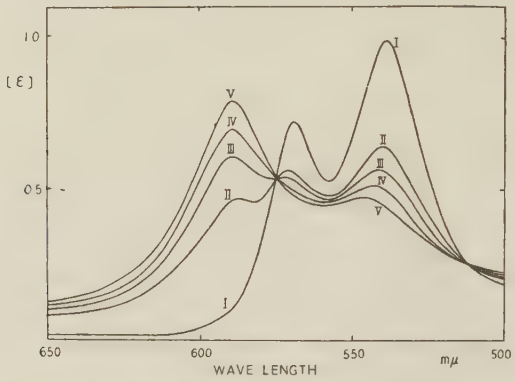


FIG. 1. The absorption change during the course of the reaction process.

Dicyanheme	5×10^{-5} *M*	3.0 ml.
Ascorbic acid	0.5 *M*	0.3 ml.
H_2O_2	0.15 *M*	0.15 ml.

I. 0 minute, II. 3 minutes, III. 20 minutes, IV 45 minutes, V. 80 minutes, Temp. 25°

measured. The peak at 538 and 568 mμ of dicyanheme decreased as the reaction proceeded and a new absorption at 588 mμ appeared instead. Its β-band was found at 545–550 mμ. Under the condition indicated in Fig. 1, the reaction was in completion within 80 minutes. Throughout the reaction process, two clear-cut isosbestic points were demonstrated at 571 and 513 mμ as an indication that the reaction takes place fairly quantitatively.

A similar absorption change was found in this reaction process also at the Soret region as illustrated in Fig. 2 including two isosbestic points at 446 and 404 mμ.

(*B*) Taking the concentrations of hemin and ascorbic acid at constant as indicated in Fig. 3, H_2O_2 was added increasingly. After

20 minutes of the reaction, the absorbance of the reaction solution was measured after reduction with dithionite. Within a certain limit of H_2O_2 concentration, the absorbance of dicyanheme decreased with the increase of H_2O_2 concentration added, as illustrated in Fig. 3, similarly

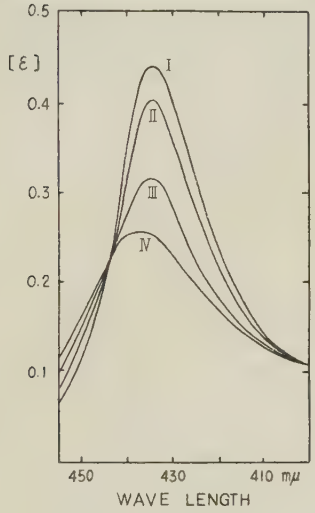


FIG. 2. The absorption change in the Soret region.

Dicyanheme 5×10^{-5} *M* 3.0 ml.
Ascorbic acid 0.5 *M* 0.3 ml.
H_2O_2 0.15 *M* 0.15 ml.
I. 0 minute, II. 2 minutes, III. 7 minutes, IV. 80 minutes, Temp. 25°

The optical density of the reaction solutions were measured after being diluted 14 times.

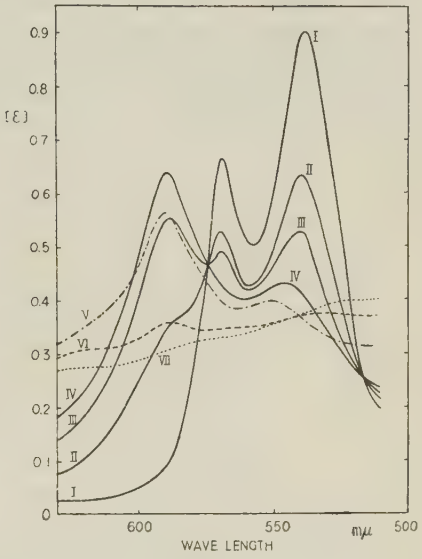


FIG. 3. Absorption change during the reaction with H_2O_2 in varying concentrations.

Dicyanheme 5×10^{-5} *M* 3.0 ml.
Ascorbic acid 0.5 *M* 0.3 ml.
H_2O_2 0.15 ml.
I. 0 *M*, II. 0.03 *M*, III. 0.09 *M*, IV. 0.15 *M*, V. 0.4 *M*, VI. 0.6 *M*, VII. 0.8 *M*, Temp. 25°

All of the absorption figures were recorded 30 minutes after the reaction.

to the former reaction as shown in Fig. 1. Further increase of H_2O_2 resulted, however, a deformation of the absorption figure in which the optical density at red region is high in general with lower density at 588 mμ.

Now, the increase in the optical density at 588 mμ was plotted against the concentration of H_2O_2 added as illustrated in Fig. 4. At the H_2O_2 concentration up to 1×10^{-2} *M*, the increase in the optical density at 588 mμ after a definite reaction time was found to correspond to the increase in the concentration of H_2O_2. Within this concentration range of H_2O_2 applied, the formation of 588 mμ compound proceeded quantitatively. Further increase of H_2O_2 concentration resulted a decrease in the optical density at 588 mμ and this was accompanied by an increase of the absorbance at the red, suggesting a more progressed reaction is taking place. Above 2.0×10^{-2} *M* H_2O_2, various side reactions

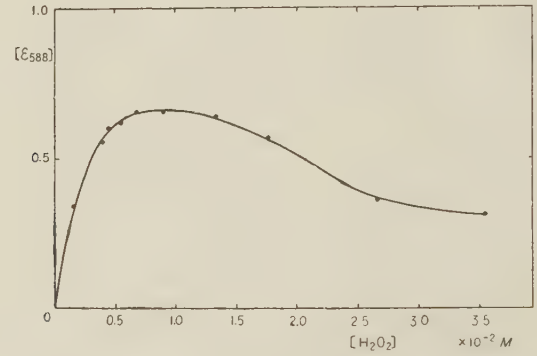


FIG. 4. Relationship between the yield of 588 mμ substance and the concentration of H_2O_2 added.
Dicyanheme 4.35×10^{-5} *M*
Ascorbic acid 4.35×10^{-2} *M*
H_2O_2 in varying concentrations as given in the figure. Temp. 25°
ε_{588} was recorded after 30 minutes of the reaction.

seemed to occur as suggested by the curves shown in Fig. 4 and by the absorption figures illustrated in Fig. 3.

(*C*) The next experiment was performed with varying concentrations of ascorbate taking the H_2O_2 concentration at constant. As shown in Fig. 5, when the concentration of ascorbic acid is low, the optical density at 588 mμ decreases and no isosbestic point can be demonstrated; it can be assumed thus that under this condition various side reactions are taking place toward the decomposition of heme. So it seems to be essential in order to maintain the mentioned reaction in an

ordinary process, an efficient concentration of ascorbate to be added to the system.

(*D*) In a similar meaning, it seems to be essential for the ordinary reaction also to keep KCN in an efficient concentration in the system. From the experiment conducted with varying concentrations of KCN, namely, with 0.125, 0.25, 0.375 and 0.5 *M* of its final concentrations, it was observed that the formation of 588 mμ compound increased with increasing KCN until its formation reached finally to a maximum limit. When K_2CO_3 was used instead of KCN and the latter was added after

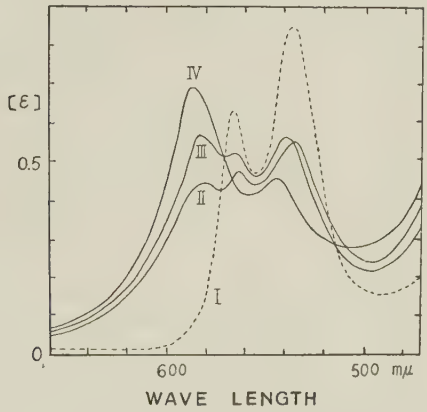


Fig. 5. Effect of abscoric acid concentration on the reaction as recorded by the absorption change.

Dicyanheme	4.35×10^{-5} *M*
H_2O_2	0.95×10^{-2} *M*
Ascorbic acid	I. dicyanheme, II. 0.0025 *M*, III. 0.005 *M*, IV. 0.05 *M*.

that the reaction passed, no 588 mμ compound could be formed.

(*E*) By undertaking the experiment in prolonged reaction time under the condition described in Fig. 1, the increase in the optical density at 588 mμ was in completion within about 80 minutes and then it began to decrease very slowly accompanied by a slow arise of an absorbance at about 610–620 mμ (Fig. 6). This reaction was completed within 24 hours. The second compound thus formed from 588 mμ compound showed its absorption maximum at 618 mμ. The reaction proceeded, however, more fast when pH of the solution was lowered.

II. Dicyanhematin—Ascorbic Acid—Oxygen System

Similar compounds as described in the preceeding section were formed also from the reaction system of dicyanhematin, ascorbic acid and atmospheric oxygen instead of H_2O_2 and with more prolonged reaction time. As indicated in Fig. 6, optical density increased at 588 mμ at first, and then followed the increase at red region. In this case, however, the reaction rate was very low, so that about 48 hours were required for the completion of the formation of 588 mμ substance. When

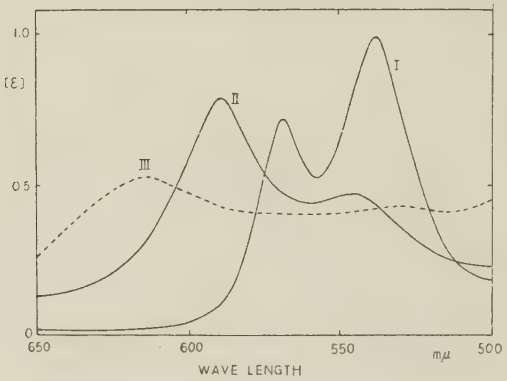


FIG. 6. Decomposition of dicyanheme to 618 mμ substance through an intermediate, 588 mμ substance.

Dicyanheme	5×10^{-5} *M*	3.0 ml.
Ascorbic acid	0.5 *M*	0.3 ml.
H_2O_2	0.15 *M*	0.15 ml.

I. dicyanheme,
II. recorded after 80 minutes of the reaction,
III. recorded after 24 hours of the reaction.

this reaction solution was further allowed to stand in air, there arises an absorption at 618 mμ.

From the observations above described in section I and II, it may plausibly be assumed that in the present reaction system, a decomposition product which is characterized by an absorption at 618 mμ is formed from dicyanheme (its absorption maxima were at 538 and 568 mμ) through an intermediate characterized by its absorption at 588 mμ.

Dicyanheme $\longrightarrow$ 588 mμ compound $\longrightarrow$ 618 mμ compound.

III. Some Properties of the Reaction Products

Thus, the present authors have identified in the mentioned reaction system, two characteristic, so far unknown decomposition product of protoheme. In order to investigate the properties of these substances, the cleaved heme portions were extracted in their free states from the reaction mixture as follows.

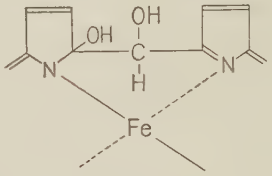
(*A*) *The Compound with its Absorption at 588 mμ*—To 100 ml. of dicyanhematin solution of 5×10^{-5} *M* (final concentration), 10 ml. of 0.5 *M* ascorbic acid and 5 ml. of 0.15 *M* H_2O_2 were added and allowed to stand in room temperature. After an hour and after that the formation of 588 mμ compound came to completion, 30 ml. of glacial acetic acid was added to the reaction solution, HCN was deriven out and was shaken with 40 ml. of chloroform for about one hour. The coloured substance was extracted thus in chloroform. The chloroform phase was washed with water about 5 times until CN^- is completely removed. The coloured compound was extracted from its chloroform solution with 10 ml. of *N*/10 NaOH. The compound which is now free from CN^- showed no more specific absorption. But, when KCN was added, there arose a diffuse absorption in the green at 500–650 mμ. The solution showed thus no characteristic absorption. When this solution was reduced with dithionite, however, there arose a perfectly similar absorption to that of 588 mμ substance above mentioned. And when, instead of KCN, pyridine or globin was added followed by reduction with dithionite, then an absorption at 580 mμ and 585 mμ respecitively, was formed. Each of these compounds did not show any absorption change on bubbling with CO-gas.

(*B*) *The Second Product, 618 mμ-Compound*—The second product was obtained from the mentioned reaction system when the reaction was conducted at pH 9.2 and with prolonged reaction time. The second product was isolated by the similar procedure as for the isolation of the first product after removal of KCN, extracting in chloroform and then in NaOH. When KCN was added to this solution, the optical density increased within the whole range. When, however, this solution containing KCN was reduced with dithionite, there arose an absorption showing a distinct maximum at 618 mμ. When pyridine was added instead of KCN followed by reduction with dithionite, an absorption maximum at 608 mμ became visible.

DISCUSSION

For the pseudohematin obtainable from a reaction system of dicyanhematin-dithionite-H_2O_2, Lemberg proposed the following formula.

The two products obtained in the present experiment seem, from their optical properties, to bear chemically a close resemblance to pseudohematin. But the first product with its absorption maximum at 588 mμ is to be assumed as a precursor of pseudohematin. A well known intermediate of the reaction of verdohemochrome formation, namely a substance showing its absorption at 630 mμ is easily autoxidizable in its pyridine solution. The new decomposition product, 588 mμ compound is, however, stable against atmospheric oxygen even in its pyridine solution. Thus the heme of 588 mμ substance is different from that of 630 mμ substance. The heme of 588 mμ compound newly found is, as one of the specific substances, in a clear distinction to the other known intermediate of heme decomposition, thus suggesting a complicated nature of the reaction of heme decomposition in general.



Pseudohematin, when boiled in NaOH alkaline solution after dithionite reduction, reversed into red hemochrome to a certain extent. The second product obtained by the authors does not reverse by the similar treatment. There is thus yet some doubt if the chemical structure of this compound coincides with that of pseudohematin.

SUMMARY

1. When dicyanhematin reacted with H_2O_2 or with atmospheric oxygen in the presence of ascorbic acid, a product was obtained which is characterized by its absorption at 588 and 545–550 mμ in its ferrous state. When the oxidation proceeded further, it gave rise of a second product which shows an absorption maximum at 618 mμ in its ferrous state.

2. Both products in their free state can combine with pyridine or globin. It is notable that their pyridine compounds possess their absorption maxima rather at shorter wavelength side compared with those of their CN-compounds. But, in respect to the absorption figure as a whole, both compounds, pyridine and cyanide compound are in close resemblance.

3. Both products are assumed to have the original closed ring structure. The second product bears a close resemblance to pseudohematin in their property though not in perfect identity. Both are stable in alkaline solution; they are not easily oxidizable by atmospheric oxygen.

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REFERENCES

(*1*) Lemberg, R., *Ann. d. Chem.*, **499**, 25 (1932)
(*2*) Fischer, H., and Lindner, F., *Z. Physiol. Chem.*, **153**, 54 (1926)
(*3*) Barkan, G., *Z. Physiol. Chem.*, **171**, 179 (1927)
(*4*) Lemberg, R., *Biochem. J.*, **29**, 1322 (1935)
(*5*) Siedel, W., *Fortschr. d. Chem. Org. Naturstoffe*, **III**. 81, Julius Springer, Wien (1939)
(*6*) Kaziro, K., *J. Jap. Biochem. Soc.*, **16**, 12 (1941)
(*7*) Lemberg, R., and Legge, J. W., Hematin Compounds and Bile Pigments, p. 456, 471, 530, Interscience Publishers, New York (1949)
(*8*) Kaziro, K., and Kikuchi, G., *Ketsuekigaku Togikai-hokoku*, **4**, 250 (1951)
(*9*) Willstätter, R., *Ann. d. Chem.*, **373**, 232 (1910) ; **385**, 197 (1911)
(*10*) Makita, M., *J. Jap. Biochem. Soc.*, **26**, 29 (1954)

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OXIDATION-REDUCTION POTENTIAL OF PROTEINASE-MODIFIED CYTOCHROME C

Sirs;

Studies on the oxidation-reduction potential of cytochromes have contributed important data toward the solution of numerous problems of biological oxidations. Recently several cytochromes have been newly discovered and their potentials measured. Of these pigments, cytochrome c_3, cytochrome f and "*R. rubrum* cytochrome c" have the same heme part attatched by thioether linkage as that of cytochrome c, but their oxidation-reduction potentials are quite different from that of cytochrome c.

TABLE I

Estimation of the Potential of Hemopeptide by Using Oxidation-Reduction Indicators (pH. 7.0)

+ ; Appearance of absorption band. ++ ; Full development of absorption band. Figures in brackets represent potentials of mixtures.

Oxidation-reduction indicator	Per cent reduction of dye				
	25%	50%	75%	90%	95%
Phenosafranine	− (−0.24)	− (−0.25)	− (−0.27)	− (−0.28)	± (−0.29)
Neutral red	++ (−0.31)	++ (−0.325)	++ (−0.34)	++ (−0.355)	++ (−0.365)

Studying the relations between the function and the protein structure of cytochrome c, the authors were interested in these differences. As the differences may be attributed to the differences of the protein parts, authors intended to measure the potentials of variously modified cytochrome c.

As "pepsin-modified cytochrome c" has been studied well, the potential of this hemopeptide (*1*) was measured and estimated to be approximately −0.3 volt by the dye method (*2*) (Table I). Similar results were obtained with trypsin-modified as well as chymotrypsin-modified cyto-

chrome c. These values are lower than the oxidation-reduction potentials of hemochromogens and cytochromes, such as histidine hemochromogen (−0.13 volt) and cytochrome c_3 (*3*) (−0.20 volt) which has the lowest potential ever known and has the same prosthetic group as cytochrome c.

The similarities are suggested between digested cytochrome c and cytochrome c_3, in potential, autoxidizability, hydroxylamine reductase activity and other properties. Recently Henderson and Rawlinson (*4*) reported that cytochrome c treated with trichloroacetic acid is 0.05 volt higher than that of unmodified cytochrome c. This value is comparable with that of "*R. rubrum* cytochrome c". By modification of protein part, "cytochrome c" of various oxidation-reduction potential may be obtained and accordingly precautions to avoid modifications will be necessary in preparation of cytochrome components, especially when process of autolysis or enzymatic digestion is included in the preparation procedures.

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REFERENCES

(*1*) Tuppy, H., and Paléus, S., *Acta Chem. Scand.*, **9**, 353 (1955)
(*2*) Yoshikawa, H., *J. Biochem.*, **38**, 1 (1951)
(*3*) Ishimoto, M., Koyama, J., and Nagai, Y., *J. Biochem.*, **41**, 763 (1954)
(*4*) Henderson, R. W., and Rawlinson, W. A., *Nature*, **177**, 1180 (1956)

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